REMARKS

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Descriptive support for the limitation of "exclusively binding," in the above amendment to claim 1 is found in the specification at pg. 15, lines 28-30. Applicant submits that no new matter is added by the above amendment and respectfully requests that the amendment be entered.

Applicant wishes to point out that in applicant's response mailed March 3, 2004, claim 3 contains a typographical error in the recitation "SEQ ID No. 1". "SEQ ID No. 1" in claim 3 had been amended to "SEQ ID No: 1" in applicant's prior response, mailed May 22, 2003. This error necessitates the current amendment of "SEQ ID No. 1" back to the previously amended SEQ ID No: 1.

The rejection of claims 1-5, 12, 13, 20, and 32-34 under 35 U.S.C. § 112 (1st para.) for lack of enablement is respectfully traversed in view of the above amendments and the following remarks.

In rejecting claims 1-5, 12, 13, 20, and 32-34 under 35 U.S.C. § 112 (1st para.) for lack of enablement, the U.S. Patent and Trademark Office ("USPTO") uses U.S. Patent No. 5,773,572 to Fishleigh et al. ("Fishleigh"), and U.S. Patent No. 6,261,790 to O'Rourke ("O'Rourke") to support its position that the specification is not enabling for any antibodies other than the monoclonal antibody produced by the hybridoma CNCM-I-2476 disclosed in the present invention. The USPTO fails to appreciate that the present invention teaches a method that has achieved the result desired (but not reached) by Fishleigh and O'Rourke, because the claimed antibody was made using different methods, and to some extent even different materials, than those taught by Fishleigh and O'Rourke. In addition, applicant submits that the making of the claimed invention is taught in such complete and sufficient detail in the present application that a skilled scientist, having read the instant application, would be fully able to make and use the claimed invention.

In particular, the USPTO states that Fishleigh and O'Rourke "immunize animals with peptides that the ordinary artisan would predict to have the essential 3-dimensional structure of SEQ ID NO: 1 or 2, yet the antibodies produced do not meet the requirement of binding the disease specific form." On the contrary, as described in detail in applicant's previous response, Fishleigh and O'Rourke use immunogenic peptides of the

PrP^{Sc} protein that differ in length and composition from that used to make the present invention. Applicant submits that one of ordinary skill in the art would reasonably assume that each additional amino acid at the C- and/or N-terminal of the peptide(s) will influence the three dimensional structure of such peptides. Furthermore, it should also be considered that the applied peptides are degraded by the cellular machinery to give either decomposed material not immunogenic or immunogenic peptide(s) eliciting an immune response. The longer a peptide, the more prone it is to intracellular degradation. Accordingly, since Fishleigh and O'Rourke provide peptides differing from those used in the present invention, and, thus, constitute different lengths and different three dimensional conformations, it would be easily understood by one of skill in the art why the disclosures of Fishleigh and O'Rourke could not provide antibodies discriminating between disease and normal forms of prion protein. For this reason, the failures of Fishleigh and O'Rourke do not evidence non-enablement of the present invention.

Furthermore, applicant submits that the instant application teaches how to make the claimed invention with sufficient detail that one skilled in the art, having read the present application, would be fully able to make and use the invention.

Applicant acknowledges that numerous attempts have been made by researchers to make an antibody against PrP^{Sc}, by immunizing laboratory animals (e.g., mice or rabbits) with protein. It was found that, using the usual methods of antibody production, preparation of an antibody against PrP or PrP^{Sc}, respectively, was not an easy task to achieve, since the animals themselves have a PrP protein (which shows a substantial homology over the species); thus, the immune system normally suppresses any immune response raised against an/the (auto-) antigen.

Normally, when immunizing laboratory animals with such peptides, no sufficient immune response will be elicited unless an adjuvant is used (e.g., Freund's incomplete adjuvant, "IFA") to support the establishment of an adequate immune response in the immunized animal. In making the present invention, the inventors chose KLH, a carrier, which is known to support the generation of an acceptable immune response (Example 1), in addition to IFA. However, as the instant application teaches, this carrier was not linked to the peptide(s) in the usual way (via the C-terminus), but instead via the N-terminus (Example 1). It will be appreciated by one skilled in the art that, in a given polypeptide, where the sequences adjacent to a given peptide sequence will have an impact on the three-dimensional conformation, the same might well apply when linking a peptide to a carrier.

While carrying out the experiments leading to the present invention, the applicant found that the sequence recited in claim 1, i.e., the peptide in essentially free form, has the same tertiary structure as it has when present in the PrP^{Sc} prion protein (but not when present in the PrP protein). That is, the peptide does not have the tertiary structure as it has in the "normal" PrP protein, but as it exhibits in the PrP^{Sc} protein (which is presently believed to involve a transition from an α-tertiary structure to a β-sheet conformation during transition from PrP to PrP^{Sc}). Without wishing to be bound to any theory, that the peptide takes on this special conformation might be due to the combination of the selection of the peptide having amino acid SEQ ID NO:1, a selection of its length, and the fact that it has a linkage via the N-terminus to KLH, the carrier, rather than the usual C-terminal linkage. In any case, the antibody raised against the said peptide is capable to distinguish between PrP and PrP^{Sc}, since it obviously recognizes/is directed to only the stretch of amino acids in the PrP protein, the tertiary structure of which changes during transition from PrP to PrP^{Sc} , and recognizes only the changed structure (i.e., the β-sheet configuration).

All questions of enablement are evaluated against the claimed subject matter (MPEP § 2164.08 (Rev. 2, May 2004)). As concerns the breadth of a claim relevant to enablement, the only relevant concern should be whether the scope of enablement provided to one skilled in the art by the disclosure is commensurate with the scope of the protection sought by the claims (MPEP § 2164.08 (Rev. 2, May 2004), citing *AK Steel Corp. v. Sollac,* 344 F.3d 1234, 1244, 68 USPQ2d 1280, 1287 (Fed. Cir. 2003); *In re Moore,* 439 F.2D 1232, 1236, 169 USPQ 236, 239 (CCPA 1971)). Applicant submits that the instant application teaches in great detail how to make the claimed antibody, including disclosing specific amino acid sequences suitable for use as antigenic peptide(s) against the PrP^{Sc} isoforms (SEQ ID NO: 1 and SEQ ID NO: 2) (Example 1); an effective adjuvant/carrier and a preferred peptide-carrier linkage for the antigen to ensure a strong immune response (Example 1); a method of immunizing animals with the peptide-carrier and an adjuvant (Example 2); the production of monoclonal antibody-producing hybridoma cell lines (Example 3); and the testing of the monoclonal antibodies for binding to PrP^{Sc} vs. PrP^{C} isoforms (Example 4). If one carries out the teaching of the instant application, the result will be an antibody that "binds exclusively to a PrP^{Sc} isoform of the prion protein" and "recognizes an epitope having the three dimensional conformation provided by a protein having the amino acid sequence of SEQ ID No. 1 (Cys-Ile-Thr-Gln-Tyr-Glu-Arg-Glu-Ser-Gln-Ala-Tyr-Tyr) of the PrP^{Sc} isoform of the prion protein, while not binding to the PrP^{C} isoform when both isoforms are present in a sample in

a native, non-denatured state." Accordingly, the rejection of claims 1-5, 12, 13, 20, and 32-34 under 35 U.S.C. § 112 (1st para.) for lack of enablement is improper and should be withdrawn.

The rejection of claims 1, 2, 4, 5, 12, 13, 20, and 32-34 under 35 U.S.C. § 102(b) as anticipated by U.S. Patent No. 5,846,533 to Prusiner et al. ("Prusiner") is respectfully traversed in view of the above amendments and the following remarks.

It is the position of the USPTO that the antibodies of Prusiner anticipate the present invention because Prusiner teaches antibodies asserted to be capable to distinguish PrP^{Sc} vs. PrP^{C} isoforms "*in situ*." For the reasons presented below, applicant disagrees.

Prusiner teaches antibodies that specifically bind to the non-denatured infectious prion protein PrP^{Sc} and can be used to assay a sample, which has any PrP^{C} denatured via proteinase K, for the presence of PrP^{Sc} of a specific species, which PrP^{Sc} is associated with disease (*see* Prusiner Abstract). Applicant submits that Prusiner teaches antibodies that are highly specific, but not exclusive, for PrP^{Sc}.

In particular, it should be noted that when discussing antibody specificity, Prusiner teaches that "antibodies for PrP^{Sc} are preferably immunospecific, i.e., not substantially cross-reactive with related materials" (*see* col. 9, lines 8-9). Additionally, antibody binding to its epitope of the specific polypeptide may be stronger than binding of the same antibody to any other epitope (*see* col. 9, lines 29-30) and specific antibodies may be capable of binding other polypeptides at a weak, yet detectable level of 10% (*see* col. 9, lines 37-40). Moreover, the antibody of Prusiner contemplates a multivalent antigen assuming more than one specific antigenic/immunogenic epitope (*see* col. 15, lines 23-25). Given these disclosures, one skilled in the art would understand that the antibodies of Prusiner are *specific* for PrP^{Sc} (i.e., have greater affinity for the PrP^{Sc} vs. PrP^{C} isoform), but that Prusiner does not teach any antibody that binds *exclusively* to the PrP^{Sc} isoform.

As further support for applicant's position that the rejection over Prusiner is improper, attached hereto is a reference that clearly supports the conclusion that the antibodies of Prusiner do not bind exclusively to PrP^{Sc}. Williamson et al., "Mapping the Prion Protein Using Recombinant Antibodies," *J. Virology* 72(11):9413-9418 (1998) ("Williamson")(Exhibit 1), describes the making and testing of the anti-PrP antibodies disclosed in Prusiner, including, but not limited to, monoclonal antibodies D4, R2, D2, D14, R1, and R10 (*see* Table 1 and Table 2 at pg. 9414). Williamson discloses that the none of the antibodies rescued from mice immunized with infectious PrP 27-30 preparations exclusively

recognized the infectious (i.e., PrP^{Sc}) form of the protein. In fact, they all reacted with cell-surface PrP^{C} (native PrP^{C}) and/or immunoprecipitated PrP^{C} from transfected CHO cells (*see* pg. 9417, rt col., 1st full paragraph, and Tables 2 and 4). Furthermore, Williamson notes that all the antibodies made detected PrP^{Sc} *in situ*, but only following a denaturation treatment of the sample (*Id.* at pg. 9417, rt. col., 1st full paragraph). Based on the disclosure of Williamson, it is evident that Prusiner does not teach an antibody capable of exclusively binding to PrP^{Sc} in a sample in a native, non-denatured state.

Because Prusiner does not teach the presently claimed invention, the anticipation rejection over Prusiner is improper and should be withdrawn.

The rejection of claims 1, 2, 3, 10, 13, 20, and 32-34 under 35 U.S.C. § 102(b) as anticipated by Korth et al., "Prion (PrP^{Sc})-Specific Epitope Defined by a Monoclonal Antibody," *Nature* 390:74-77 (1997) (Korth I), is respectfully traversed in view of the above amendments and the following remarks.

It is the position of the USPTO that Korth I anticipates the present invention because the 15B3 antibody of Korth I is capable of distinguishing between PrP^{C} and PrP^{Sc} isoforms. Applicant submits, as described below, that Korth I does not meet all the limitations of the present invention as amended.

Korth I describes the production of a monoclonal antibody (15B3) against PrP, obtained by immunizing PrP-null mice with the full length recombinant bovine PrP. Korth I further discloses that 15B3 recognizes a conformational epitope constituted by three different stretches of amino acids in bovine rPrP, namely 15B3-l, l5B3-2 and 15B3-3, which are distributed over the C-terminal half of the PrP^{Sc} isoform of the prion protein. 15B3-l, l5B3-2 and 15B3-3 correspond to amino acids 142-148 ("first segment"), 162-170 ("second segment"), and 214-226 ("third segment"), respectively, of the recombinant bovine PrP. The third segment of bovine PrP corresponds to SEQ ID No. 2 of the present invention, and the related third segment of human PrP corresponds to SEQ ID No. 1 of the present invention.

First of all, 15B3 binds to a conformational epitope constituted by three non-contiguous amino acid sequences of recombinant bovine PrP. Thus, 15B3 binds to a three-dimensional structure made up of the three peptide sequences, and all three peptide sequences contribute to the binding affinity of the 15B3 antibody. It will be appreciated that the antibody will also bind to an epitope made up of only two of the sequences, though with a lower affinity. Therefore, Korth I does not teach a monoclonal antibody or fragment thereof

that "is directed essentially to a peptide having the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2."

In addition, applicant has noted that in actual practice 15B3 (which is commercially available and used by those in the art) has a *preference* for PrP^{Sc}, but does not bind "exclusively to a PrP^{Sc} isoform of the prion protein"; nor does 15B3 "recognize[] an epitope...of the PrP^{Sc} isoform of the prion protein while not binding to the PrP^{C} isoform when both isoforms are present in a sample in a native, non-denatured state." These observations are supported by the fact that in carrying out the PrP^{Sc} detection assay using mAb 15B3, proteinase K must always be used. Therefore, 15B3 does not bind "exclusively to a PrP^{Sc} isoform of the prion protein," as evidenced by the need to manipulate binding conditions appropriately to avoid binding to any PrP^{C} isoform present in the sample. Applicant's position that 15B3 does not bind to PrP^{Sc} *exclusively* is also supported by the observations of others skilled in the art. For example, it was noted recently that early claims of antibodies capable to discriminate between the PrP^{C} and PrP^{Sc} isoforms, "such as Prionics' 15B3 clone (Korth et al., 1997), have not lived up to the expectations." Aguzzi et al., "Review: Mammalian Prion Biology: One Century of Evolving Concepts," *Cell* 116:313-327 at 315, right column (2004)) (attached hereto as Exhibit 2). Thus, applicant submits that, while the antibody of Korth I may be highly specific for the PrP^{Sc}, it does not bind "exclusively to a PrP^{Sc} isoform of the prion protein"; nor does it "recognize[] an epitope...of the PrP^{Sc} isoform of the prion protein while not binding to the PrP^{C} isoform when both isoforms are present in a sample in a native, non-denatured state."

For all the above reasons, Korth I cannot anticipate the present invention, and the anticipation rejection over Korth I should be withdrawn.

The rejection of claims 1, 2, 3, 5, 10, 12, 13, 20, and 32 under 35 U.S.C. § 102(b) as anticipated by EP 0 861 900 A1 to Korth et al. ("Korth II"), is respectfully traversed.

Korth II discloses the same monoclonal antibody, i.e., mAb 15B3, as Korth I. Therefore, Korth II cannot anticipate the present invention as amended for substantially the same reasons as described in the discussion of the rejection over Korth I, above. The rejection of claims 1, 2, 3, 5, 10, 12, 13, 20, and 32 under 35 U.S.C. § 102(b) as anticipated by Korth II is also improper and should be withdrawn.

In view of all of the foregoing, applicant submits that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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Mapping the Prion Protein Using Recombinant Antibodies

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The fundamental event in prion disease is thought to be the posttranslational conversion of the cellular prion protein (PrP^C) into a pathogenic isoform (PrP^{Sc}). The occurrence of PrP^C on the cell surface and PrP^{Sc} in amyloid plaques in situ or in aggregates following purification complicates the study of the molecular events that underlie the disease process. Monoclonal antibodies are highly sensitive probes of protein conformation which can be used under these conditions. Here, we report the rescue of a diverse panel of 19 PrP-specific recombinant monoclonal antibodies from phage display libraries prepared from PrP deficient ($Prnp^{0/0}$) mice immunized with infectious prions either in the form of rods or PrP 27-30 dispersed into liposomes. The antibodies recognize a number of distinct linear and discontinuous epitopes that are presented to a varying degree on different PrP preparations. The epitope reactivity of the recombinant PrP(90-231) molecule was almost indistinguishable from that of PrP^C on the cell surface, validating the importance of detailed structural studies on the recombinant molecule. Only one epitope region at the C terminus of PrP was well presented on both PrP^C and PrP^{Sc}, while epitopes associated with most of the antibodies in the panel were present on PrP^C but absent from PrP^{Sc}.

Prion diseases are disorders of protein conformation that are characterized by a profound degeneration of the central nervous system (24, 25). The fundamental event in the pathogenesis of these diseases is the conversion of the cellular prion protein (PrP^C) into the scrapie isoform (PrP^{Sc}). Evidence from modeling structural studies, including infrared spectroscopy, circular dichroism, and multidimensional heteronuclear solution nuclear magnetic resonance (NMR) argues that PrP^{Sc} formation involves an extensive conformational change in which the α-helical content of PrP diminishes and a large amount of β-sheet is acquired (3, 6, 11, 13, 19, 21, 28, 31, 35). Detailed structural studies of PrP^{Sc} have, however, been technically difficult to carry out. Limited proteinase K digestion employed during the purification of PrP^{Sc} yields PrP 27-30 which assembles into rod-shaped polymers with the ultrastructural and tinctorial properties of amyloid (18, 27).

Another approach to probing conformational transitions in prion proteins is to generate antibodies to diverse epitopes of PrP^C and PrP^{Sc}. However, natural infection induces no humoral immune response to infectious scrapie particles (17), and immune tolerance to the highly conserved PrP amino acid sequence has restricted the generation of monoclonal antibodies in normal mice (2, 15, 30). To access a wider spectrum of PrP-specific monoclonal antibodies, we raised antisera recognizing mouse (Mo) and Syrian hamster (SHa) PrP in mice homozygous for PrP gene knockout ($Prnp^{0/0}$) (4, 26) and prepared combinatorial phage antibody libraries from these animals as described previously (1, 5, 12, 34).

Antibody libraries were constructed from $Prnp^{0/0}$ mice immunized either with prion rods containing MoPrP 27-30 or with disaggregated PrP 27-30 incorporated into liposomes (9, 10, 22). Mice immunized with prion rods received an immunization and three boosts. Animals immunized with PrP 27-30 in liposomes were divided into two groups and received either an immunization and two boosts (long immunization) or, in an attempt to increase the diversity of the antibody response, an immunization and a single boost (short immunization). For each mouse, PrP-specific reactivity in all four subclasses of serum immunoglobulin G (IgG) was determined by enzyme-linked immunosorbent assay (ELISA) against MoPrP 27-30 treated with the denaturant guanidium thiocyanate (GdnSCN). Mice immunized with prion rods generated PrP-specific serum antibody titers predominantly in the IgG1 and IgG2b subclasses, whereas mice immunized with PrP 27-30 liposomes produced a strong PrP-specific response in all IgG subclasses. Serum antibody reactivity has proven to be accurate in predicting the specificities rescued from the corresponding phage libraries (5, 33). We therefore prepared an IgG1κ and an IgG2bκ Fab library from a mouse immunized with prion rods. Additional IgG1κ, IgG2aκ, IgG2bκ, and IgG3κ Fab libraries were individually constructed from each of the two groups of mice given long and short immunizations with PrP liposomes. All of the libraries were prepared with total RNA extracted from spleen, bone marrow, and lymph node tissue, and all contained over 10^7 members.

The phage libraries were individually selected against denaturant-treated PrP 27-30, recombinant PrP(90-231) and detergent dispersed PrP 27-30 as previously described (1, 22). Phage recovered from the fourth or fifth round of panning were converted to express soluble Fab (1) and tested for specific PrP reactivity in ELISA against denaturant treated PrP 27-30 and SHaPrP(90-231). The heavy chain amino acid sequences were determined for antigen-reactive Fab clones, and this information allowed the clones to be sorted into distinct families, as illustrated in Table 1.

Libraries constructed from mice immunized with Mo prions yielded five novel antibodies, designated Fabs PrP28, $PrP1^{blocked}$, $PrP34^{blocked}$, $PrP3^{recPrP}$, and $PrP28^{DLPC}$ (34). Libraries con-

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TABLE 1. Partial heavy chain amino acid sequences of selected recombinant Fabs recovered from immunized mice

Clone[a]	FR3 region sequence	CDR3 region sequence	FR4 region sequence	Epitope reactivity[b]
Immunization with MoPrP 27-30 rods				
PrP28	KATLTADKSSSTAYLDLRSLTSEDSAVYFCAR	HDGYPFAY	WGQGTLVTVSA	DC
$PrP3^{recPrP}$	KATLTADKTSSTAHIQLSSLTSEDSAVYFCAR	GFYYGSRYGPMDY	WGQGTSVIVSS	DC
$PrP28^{DLPC}$	KATLTADKSSSTAYMDLRSLTSEDSAAYFCAR	VPISVY	WGQGTTLTVSS	DC
$PrP1^{blocked}$	KATLTVDKSSSTAYIQPSSLTSEDSAVYYCAR	WGPFFYYGSRPSYYAMDS	WGQGGSVTVFS	DC
$PrP34^{blocked}$	RATLTADKSSTTAHLQLFSLSSEDSAVYFCSR	SRSTNYFDY	WGQGTTLAVSS	DC
Immunization with dispersed SHaPrP 27-30 incorporated into liposomes				
R1	KATLTVDTSSSTAYVDLSSLTSEDSAVYYCAR	EGHFPPDY	WGQGTTLTVSS	III
R2	KATLTVDKSSSTAYIQLSRLTSEDSAVYYCAR	EGDAYPFGH	WVQGTLVTVSS	III
R5	KATLTVDTSSSTAYVDLNSLTSEDSAVYYCTR	EDSSYPFAY	WGQGTTLTVSS	III
R10	KATITADTSSNTVYLQLRSLTSEDTAIYYCGR	FDGNGWYFDV	WGAGTTVTVSS	I
R23	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCAR	GGYYGAMDY	WGQGTSVTVSS	DC
R25	RATLTADTSSSTAYMQLSSLTSEDSTVYFCAR	RRLITTLVDSWSFDV	WGQGTTVTVSS	DC
R40	KATLTADKSSSTAYMELRSLTSEDSAVYFCAR	DYVKGYFDV	WGTGTTVTVSS	DC
R72	EATLTVDKSSSTAYMELRSLTSEDTAVYYCVR	RGIYHYAMDY	WGQGTSVTVSS	II
D2	KATLTVDKSSSTAYMQLSRLTSEDSAVYYCAR	EGDYYPFGH	WGQGTLVTVSS	III
D4	KATITADTSSNTVYLQLRSLTSEDTAIYYCGR	FDGNGWYLDV	WGAGTTVTVSS	I
D7	RFAFSLETSASTAYLQINNLQNEDTATYFCVS	RGGDYGSSAFDY	WGQGTTLTVSS	III
D13	RFTISRDNAKNTLYLQMSSLKSDDTAMYYCGR	LGGDYGGSYLDY	WGQGTTLTVSS	I
D14	KATLTVDKSSSTAYMELRSLTSEDSAVYYCAA	YFYAMDY	WGQGTSVTVSS	DC
D18	KATLTVDKSSSTAYMELRSLTSEDSAVYYCAG	FYYGMDY	WGQGTSVTVSS	DC

[a] Clones R1, R2, R5, R10, R23, R25, R40, and R72 were panned against recombinant SHaPrP(90-231) and clones D2, D4, D7, D13, D14, and D18 were panned against dispersed SHaPrP 27-30.
[b] DC, discontinuous. I, II, and III are designations of linear epitope regions as described in the text.

structed from mice immunized with PrP liposomes initially yielded a large number of closely related sequences, of which Fabs R1, R2, R5, and R10 are examples. Fabs D2, D4, D5, D7, D13, D14, and D18 were recovered by panning against dispersed SHaPrP 27-30. On a number of occasions, Fabs with similar sequences were recovered by panning against both SHaPrP(90-231) and SHaPrP 27-30 (e.g., R2 and D2, respectively). To generate greater diversity, the PrP antigens were masked with Fabs rescued from the first panning experiments, then re-presented to the libraries (7). Amino acid sequences of ELISA reactive Fab clones taken from these experiments contained several additional Fabs, R23, R25, R40, and R72, with novel heavy chain amino acid sequences.

The antigen-binding profiles of the novel recombinant Fabs were assessed against various PrP preparations as shown in Table 2. Reactivity of Fabs with cell surface $MoPrP^C$ was assessed by flow cytometry as described previously (34), using the mouse neuroblastoma line N2a (16) and a transfected Chinese hamster ovary (CHO) cell line expressing $SHaPrP^C$. Antibody recognition of PrP^C and PrP^{Sc} in situ was examined by immunostaining blotted cryostat sections of brains taken from normal uninoculated CD-1 mice and SHa, and from clinically ill CD-1 mice and SHa inoculated with Mo(RML) prions and Sc237 prions, respectively, as described previously (32). The Fabs were also assessed for their ability to immunoprecipitate $SHaPrP^C$ from transfected CHO cells (14, 22) and SHaPrP 27-30 from liposomes (22).

All of the antibodies reacted well in ELISA with recombinant SHaPrP(90-231) and with PrP 27-30 rods following incubation with 3 M GdnSCN. All of the antibodies also detected PrP^{Sc} in situ following treatment with denaturant and, with the exception of Fab R72, also efficiently immunoprecipitated

TABLE 2. Reactivity of recombinant monoclonal Fabs against different PrP preparations[a]

Fab	Epitope reactivity	Cell-surface PrP^C	REC PrP (90-231)	PrP 27-30 in ELISA (GdnSCN treated)	PrP 27-30 in ELISA (untreated)	PrP 27-30 immunoprecipitation	PrP^C immunoprecipitation	PrP in situ (GdnSCN treated)
PrP28	DC	+	+	+	−	nd	nd	+
$PrP28^{DLPC}$	DC	+	+	+	−	nd	nd	+
$PrP3^{recPrP}$	DC	±	+	+	−	nd	nd	+
$PrP1^{blocked}$	DC	+	+	+	−	nd	nd	+
$PrP34^{blocked}$	DC	+	+	+	−	nd	nd	+
R10	I	+	+	+	−	−	+	+
R72	II	−	+	+	±	±	+	+
R1	III	+	+	+	+	+	+	+
R23	DC	+	+	+	−	−	+	+
R25	DC	+	+	+	−	−	+	+
R40	DC	+	+	+	−	−	+	+
D14	DC	+	+	+	−	−	+	+
D18	DC	+	+	+	−	−	+	+

[a] For an explanation of epitope reactivity data, see Table 1, footnote *b*. +, strong reactivity; ±, weak reactivity; −, no reactivity; nd, not done.

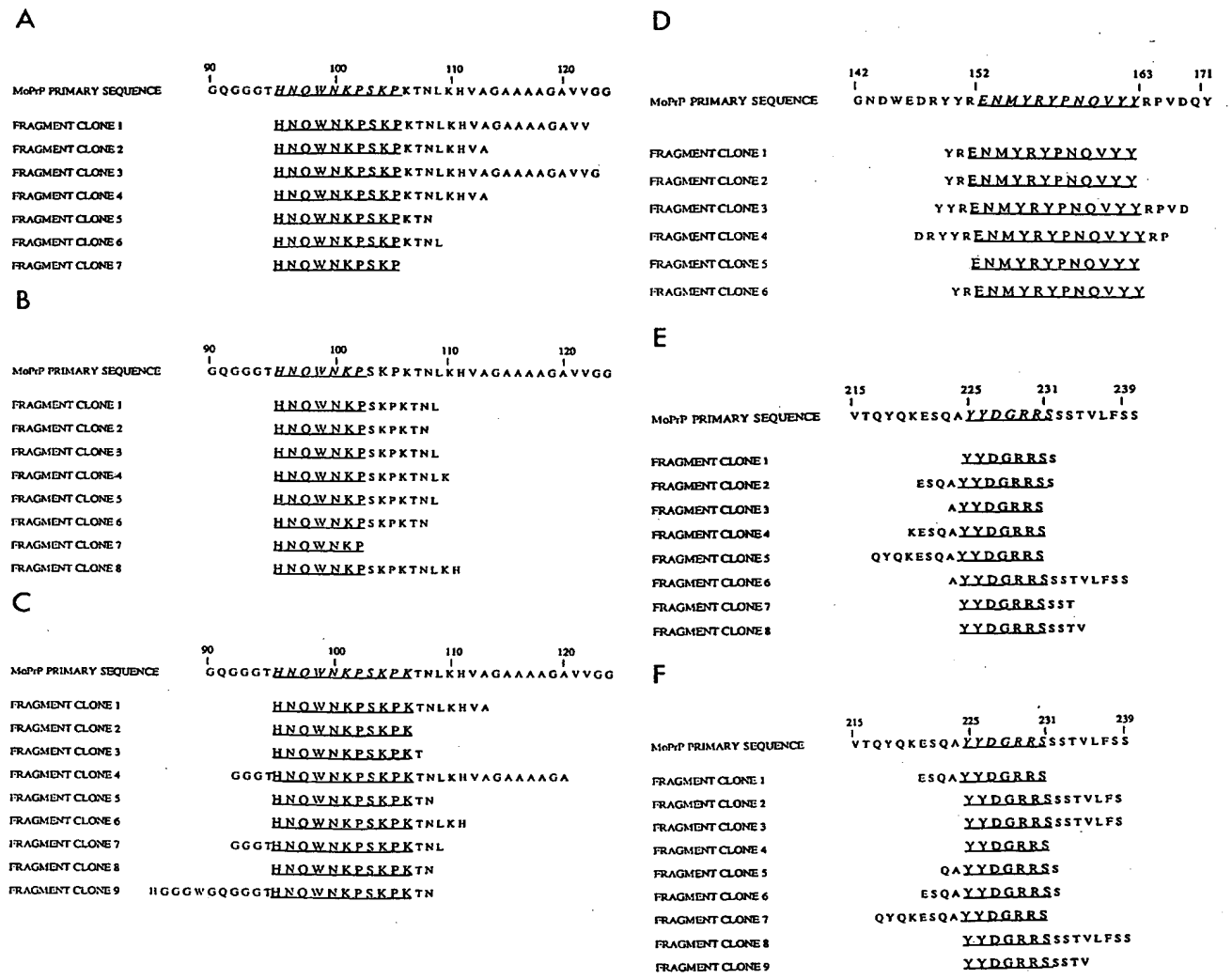

FIG. 1. Identification of linear epitopes from PrP protein fragment phage display libraries. Three linear epitopes, designated I, II, and III, were identified by panning the PrP fragment libraries against recombinant Fab fragments applied to ELISA wells. Sequence alignments of clones taken following two or three rounds of panning are shown below the corresponding mouse PrP amino acid sequence. Regions of commonality are underlined. (A) Fab R10; (B) Fab D4; (C) Fab D13; (D) Fab R72; (E) Fab R1; (F) Fab R2.

SHaPrP^C from transfected CHO cells. PrP^C in its native state on the surface of the mouse neuroblastoma line N2a (16) and a transfected CHO cell line expressing SHaPrP^C were recognized well by all Fabs, with the exceptions of PrP3^{rPrP}, which bound weakly, and R23 and R72, which did not bind at all.

We next sought to identify the binding epitopes recognized by the recombinant antibodies by using both peptide-based ELISA studies and PrP fragment libraries displayed on filamentous phage. Initially, we studied antibody reactivity against a series of synthetic peptides representing residues 90 to 231 of SHaPrP. Twenty-seven peptides that were 15 residues in length and that overlapped by 5 residues at the N terminus were prepared and individually applied to ELISA plates to determine the reactivity of each antibody. In addition, protein fragment libraries of mouse PrP were prepared for display on the surface of M13 phage via fusion with coat protein III by using the phagemid vector pFRAG in a variation of the method of Petersen et al. (23). pFRAG was constructed by placing a 39-base-pair insert containing two distinct *Bgl*II sites into the *Xho*I and *Sfi*I sites of the phage display vector pComb3 (1). The fragment libraries were panned individually over each recombinant Fab bound to ELISA wells. Following specific enrichment over sequential rounds of panning, the encoded PrP fragments of a representative population of phagemid clones were determined by DNA sequencing. Alignment of these sequences permitted the identification of a core sequence common to each clone, which likely approximates to the epitope of the antibody, as shown in Fig. 1.

Data collected by both these approaches were highly consistent and indicated that a subset of recombinant antibodies, recovered following immunization with PrP liposomes, recognized three linear epitope regions, designated I, II, and III. Epitope region I was recognized by three antibodies (R10, D4, and D13) and lies within residues 96 to 104, a region of the protein shown in solution NMR studies to be largely disordered (8, 13, 29). Epitope region II was localized to residues 153 to 161 and was bound exclusively by Fab R72. This antibody was recovered when libraries prepared from mice immu-

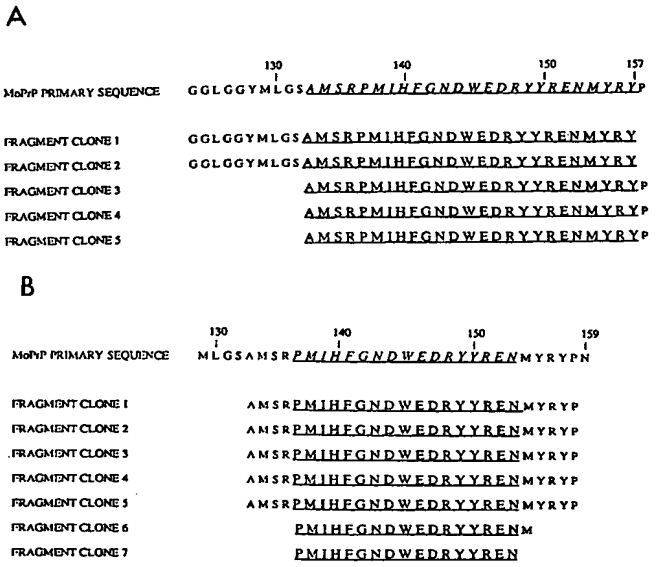

FIG. 2. Identification of nonlinear epitopes from PrP fragment display libraries. Of the recombinant Fabs that did not recognize overlapping 15-mer PrP peptides only (A) D18 and (B) R40 specifically selected for phage bearing related PrP sequence. The sequences shown were obtained following five rounds of panning. Regions of commonality are underlined.

nized with PrP liposomes were panned against recombinant SHaPrP(90-231). Its epitope contains the final four residues of the first helical region of PrP(90-231) and extends to the beginning of a short β-strand (S2) (13). Epitope region III was assigned to residues 225 to 231 at the very C-terminal end of PrP, adjacent to the glycosylphosphatidylinositol anchor. We presume this region to be immunodominant in mice immunized with PrP liposomes since the majority of Fab-phage recovered from panning experiments against SHaPrP(90-231) and dispersed SHaPrP 27-30 reacted with this epitope. Of the recombinant Fabs that did not react with short peptides, only R40 and D14 specifically enriched phage from the PrP fragment library (Fig. 2). Fab R40 isolated phage that contained the amino acid sequence between residues 138 and 155, with a minimum consensus sequence of 17 amino acids between residues 137 and 153. Fab D18 enriched for phage bearing PrP sequence containing residues 133 through 157.

To determine whether any of the Fabs would specifically bind to PrP determinants in solution, we employed a competition ELISA technique in which antibody was preincubated with a range of concentrations of competing peptides, typically 50 μM to 50 pM, before being applied to PrP antigen. Sigmoidal binding curves were obtained for each antibody competition with Graphpac (ISI Software). The concentration of each peptide required to inhibit 50% of the recombinant Fab binding to the control polypeptide applied to the plate was then determined. The results are given in Table 3. Fabs R10 and D13, possessing divergent heavy chain amino acid sequences and both binding similar if not identical epitopes between residues 96 to 104, were competed effectively with synthetic peptides corresponding to residues 90 to 104 and 95 to 109. Fabs R1, R2, and D7 recognized epitope region III (residues 225 to 231) and were efficiently competed by peptides containing amino acids 220 to 231 and 225 to 231. Interestingly, Fab R72 was efficiently competed with a peptide containing residues 152 to 163 (the region identified as the binding epitope by the fragment libraries) but did not bind at all to recombinant SHaPrP(90-231) in solution. This epitope was, however, bound tightly when SHaPrP(90-231) was applied directly to ELISA wells, indicating that the epitope is normally either partially or completely inaccessible but becomes exposed when PrP is applied to ELISA plates.

We reasoned that the antibodies recognizing discontinuous epitopes of PrP may bind longer synthetic peptides which may be able to adopt secondary structure arrangements found in the full-length protein (13, 29). We therefore synthesized a series of longer peptides corresponding to SHaPrP sequence between amino acids 90 and 145, 121 and 167, 147 and 167, 141 and 178, 159 and 201, 178 and 231 (containing protected cysteine side chains and therefore unable to form the disulfide bridge normally found between cysteine 179 and cysteine 214 in intact PrP), and 174 and 231. However, none of the recombinant Fabs was able to bind well to any of these peptides in a direct binding or competition ELISA, although recombinant SHaPrP(90-231) and SHaPrP(29-231) in α-helical states (8, 13) were bound tightly (data not shown). Similarly, in a competitive ELISA, with the exception of Fab R40, none of the Fabs was competed by the longer peptides. Fab R40 was partially competed with a peptide containing residues 127 to 167, which includes the region of sequence identified by this Fab from the protein fragment libraries (Fig. 3). We conclude that the discontinuous epitopes of PrP recognized by the antibodies may be fully formed only in the intact PrP(90-231) molecule.

To examine species cross-reactivity, the recombinant antibodies were reacted in ELISA with SHa-, Mo-, bovine, and human PrP (Table 4). Fabs binding epitope region I reacted very strongly with SHa- and MoPrP but had only very weak reactivity with bovine and human PrP. When amino acid sequences from the different species were examined in the region of epitope I, the only variation occurred at position 97, which is an asparagine residue in SHa- and MoPrP but is a serine residue in human PrP and a glycine residue in bovine PrP. The results suggest that the amino acid at position 97 makes direct contact with the group I antibodies. Epitope region II, recognized by Fab R72, is invariant across the species examined here, and predictably this antibody bound very strongly to all the PrP samples in ELISA. In contrast, residues 225 to 231 that compose epitope region III exhibit considerable diversity

TABLE 3. Inhibition of Fab binding by various PrP peptides

Fab	Concn (μM) required to inhibit 50% of Fab binding by:								
	PrP(90-231)	PrP(90-104)	PrP(96-105)	PrP(147-167)	PrP(152-163)	PrP(154-163)	PrP(155-163)	PrP(220-231)	PrP(225-231)
R10	3.0	0.8	0.4						
D13	0.06	0.1	0.08						
R72	>50			1.2	1.9	7.8	18.6		
R1	2.9							0.2	1.4
R2	0.7							1.2	0.3
D7	0.3							0.01	0.01

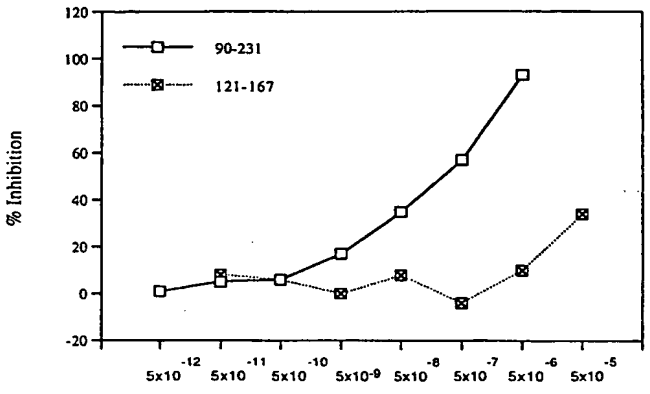

FIG. 3. Dose response of the competing antigens recombinant SHaPrP(90-231) and synthetic peptide SHaPrP(127-147) with recombinant Fab R40. Absorbance values were converted into percentages of inhibition.

across different species. Fabs recognizing this region of PrP predictably bound to SHa- and MoPrP, which contain identical sequences between residues 225 to 231, but not to PrP from the other species tested, which contain markedly different sequences in this region.

In summary, we have generated a diverse panel of PrP-specific antibodies from immunized mice. These antibodies have been characterized in terms of their amino acid sequences, the binding epitopes recognized, and their reactivity with a number of PrP-antigenic presentations. Surprisingly, given that we immunized mice with infectious PrP 27-30 preparations, none of the rescued antibodies exclusively recognized this form of the protein, whereas all but one antibody clone reacted well with PrP^C as it occurs on the cell surface. Significantly, the epitope reactivity of recombinant PrP was almost identical to that of the cell surface molecule. This finding provides direct evidence that the conformations adopted by the recombinant preparations used in structural studies of PrP closely approximate to that of PrP^C in its native state.

Only Fabs binding to epitope region III recognized PrP 27-30 prior to treatment with denaturant (22). In contrast, although available in PrP^C, epitope I was not reactive in PrP 27-30 prior to treatment with and removal of denaturant. This same pattern was observed for the antibody 3F4 which binds in the region of residues 109 to 112 (22, 30). These findings suggest that the C-terminal portion of PrP^C, which contains a highly ordered structural core composed of helices B and C, remains relatively unaltered as PrP^C is converted to PrP^{Sc}, whereas the N-terminal portion of the molecule undergoes extensive conformational rearrangement in which epitopes in the N terminus are either altered or buried in PrP^{Sc}. This conclusion is supported by protein engineering studies showing that this region of PrP is essential for PrP^{Sc} formation (20), by spectrophotometric studies which illustrate conformational plasticity in synthetic peptides corresponding to residues 90-145 (35), and by NMR studies which indicate that the N-terminal portion of PrP between residues 29 and 124 is highly flexible (8, 29).

Recombinant Fabs which did not recognize short linear amino acid sequences exhibited largely similar PrP reactivities, binding to PrP^C on the cell surface, recombinant PrP(90-231), and PrP 27-30 rods following incubation with denaturing agents. These data imply similar epitope presentation between native PrP^C, recombinant PrP(90-231), and denaturant-treated PrP^{Sc}. Hence, following denaturation in GdnSCN, presumably to a random coil state, PrP does not refold into the infective form but rather into a PrP^C-like conformation. Although the epitopes of these antibodies have yet to be identified, this study does indicate that their binding sites are highly conformationally sensitive and are probably formed from secondary and possibly tertiary structural elements of PrP.

TABLE 4. Binding of selected recombinant antibodies and the hybridoma-derived antibody 3F4 against cellular PrP^a

Antibody	Reactivity of antibody against:			
	MoPrP	SHaPrP	Human PrP	Bovine PrP
R10	+++	+++	+	+
3F4	−	+++	+++	−
R72	+++	+++	+++	+++
R2	+++	+++	−	−
R23	−	+++	+++	−
R40	+++	+++	+++	−
D14	+++	+++	+++	++
D18	+++	+++	++	+
PrP28	+++	+++	++	nd
$PrP28^{DLPC}$	+++	+++	++	nd

[a] Number of plus signs (+) indicates degree of reactivity. −, no reaction; nd, not done.

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Mammalian Prion Biology: One Century of Evolving Concepts

Review

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Prions have been responsible for an entire century of tragic episodes. Fifty years ago, kuru decimated the population of Papua New Guinea. Then, iatrogenic transmission of prions caused more than 250 cases of Creutzfeldt-Jakob disease. More recently, transmission of bovine spongiform encephalopathy to humans caused a widespread health scare. On the other hand, the biology of prions represents a fascinating and poorly understood phenomenon, which may account for more than just diseases and may represent a fundamental mechanism of crosstalk between proteins. The two decades since Stanley Prusiner's formulation of the protein-only hypothesis have witnessed spectacular advances, and yet some of the most basic questions in prion science have remained unanswered.

Introduction

A few years ago, it was memorably stated that prion diseases (also termed transmissible spongiform encephalopathies, or TSE) constitute one of the best-understood groups of neurodegenerative diseases (DeArmond and Prusiner, 1995). Depending on whom you ask, this statement may be regarded as entirely correct or completely off the mark.

Of course, prion diseases are quite well understood. Largely thanks to the enthusiasm and intuition of pioneers such as Stanley Prusiner and Charles Weissmann, progress in prion science has experienced two decades of quantum leaps. These include the isolation of the disease-associated, protease-resistant prion protein, PrP^{Sc} (Bolton et al., 1982), the formulation of the protein-only hypothesis (Prusiner, 1982), the cloning of the *Prnp* gene that encodes PrP^{C} and the startling realization that it is a normal, cellular gene (Basler et al., 1986; Chesebro et al., 1985; Oesch et al., 1985), the discovery that the host-determined aspects of the "species barrier" are crucially governed by the sequence of PrP^{C} (Scott et al., 1989), the linkage between *PRNP* mutations and hereditary prion disease (Hsiao et al., 1989), and the demonstration that PrP^{C}-deficient mice are alive and well, but resistant to prion diseases (Büeler et al., 1992, 1993).

And yet one may argue that prions are not well understood at all! We are still unable to precisely pinpoint the physical nature of the agent (Chesebro, 1998), and we do not avail of any high-resolution molecular structure of PrP^{Sc}. Hence, the models of conversion of PrP^{C} to PrP^{Sc} are speculative at best, and the conversion process could not be reproduced under cell-free conditions in a way that would lead to replication of prion infectivity. Finally, precious little knowledge is available on how the infectious agent damages the brain, and the function of the normal protein continues to be obscure.

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The Timeline of TSE Research

In one or the other form, prions have captured a sizeable mind share for almost two centuries (Table 1). Scrapie—the prototypic prion disease affecting sheep and goat—had been a concern since the 19th century. This is understandable given the importance of the wool textile business in the industrial revolution. But the crucial breakthrough was already achieved in the 1930s by the experimental transmission of scrapie to goats (Cuille and Chelle, 1939). Little happened in the two following decades, until Carleton Gajdusek showed that kuru, which was decimating the aborigines of Papua New Guinea (Gajdusek and Zigas, 1957), was a transmissible spongiform encephalopathy. Interestingly, the first attempts at transmitting kuru to primates failed for the same reason that experimental transmission of scrapie among sheep had failed for decades: the incubation time of the disease was longer than the patience of the investigators (Schwartz, 2003). Following a concise suggestion by William Hadlow that kuru resembled scrapie, and hence might exhibit a very long incubation time (Hadlow, 1959), Gajdusek achieved transmission of kuru to chimps (Gajdusek et al., 1966, 1967) and, shortly thereafter, transmission of Creutzfeldt-Jakob disease (CJD) (Gibbs et al., 1968).

It is remarkable (and somewhat sobering) to note that some of the questions that had already been formulated in the 19th century are still open. For example, is sheep scrapie a predominantly genetic or infectious disease? If the latter is true, how does it spread among flocks? The wildfire-like epizootic of chronic wasting disease in North American cervids (Williams and Young, 1980), as well as the "scrapie eradication plan" of the European Union (which aims at selective breeding of purportedly scrapie-resistant sheep genotypes), bears the most recent witness to the general importance of these issues.

The Nature of the Prion

Throughout this paper, the term "prion" is used to denote the infectious principle active in TSEs. The various hypotheses of TSE pathogenesis state that the prion may be congruent, partially overlapping, or different from the protease-resistant form of PrP found in prion diseases, which is termed PrP^{Sc}.

Two papers reprinted in the current issue of *Cell* represent two major turning points in prion research. The first paper describes the discovery, by Stanley Prusiner and coworkers, of a crucial property of the prion: its remarkable resilience against proteolytic degradation (McKinley et al., 1983). Digestion with 50 μg/ml of proteinase K (PK) at 37°C for 2 hr would not degrade the carboxy-proximal domain of PrP^{Sc} nor decrease the infectious titer of the prion preparation. But PrP^{Sc} is not "unbreakable" and can eventually be digested by more vigorous enzymatic treatment—in which case prion infectivity titers will also subside. This remarkable discovery identi-

Table 1. Essential Chronology of Prion Research

Mid 18th Century	Earliest Description of Scrapie Recorded
1898	Neuronal vacuolation discovered in brains of scrapie-sick sheep
1918	Contagious spread of scrapie in natural conditions suspected
1920	First cases of CJD described (Creutzfeldt, 1920; Jakob, 1921)
1937	Scrapie epidemic in Scotland following administration of formalin-treated louping ill vaccine prepared from sheep brain
1939	Experimental transmission of scrapie reported (Cuille and Chelle, 1939)
1955–1957	Kuru discovered among Fore people of Papua New Guinea (Gajdusek and Zigas, 1957)
1959	Similarities between kuru and scrapie noted (Hadlow, 1959)
1961	Multiple strains of scrapie agent described (Pattison and Millson, 1961)
1961	Scrapie transmitted to mice (Chandler, 1961)
1963	Transmission of kuru to chimpanzees reported (Gajdusek et al., 1966)
1966	Scrapie agent found to be highly resistant to ionizing radiation and ultraviolet light (Alper et al., 1966, 1967)
1967	First enunciation of the protein-only hypothesis (Griffith, 1967)
1968	CJD transmitted to chimpanzees (Gibbs et al., 1968)
	Description of *Sinc* gene affecting scrapie incubation period in mice (Dickinson et al., 1968)
1974	First documented iatrogenic prion transmission (corneal graft) (Duffy et al., 1974)
1980	Protease resistant, highly hydrophobic protein discovered in hamster brain fractions highly enriched for scrapie infectivity (Prusiner et al., 1980)
1982	Prion concept enunciated (Prusiner, 1982)
1985	Gene encoding PrP^{C} cloned (Chesebro et al., 1985; Oesch et al., 1985)
1986	PrP^{C} and PrP^{Sc} isoforms shown to be encoded by same host gene (Basler et al., 1986)
1987	Linkage between *Prnp* and scrapie incubation period in mice (Westaway et al., 1987)
	First report of BSE in cattle (Wells et al., 1987)
1989	Mutation in PrP linked to Gerstmann-Sträussler syndrome (Hsiao et al., 1989)
	Importance of isologous PrP^{C}/PrP^{Sc} interactions established (Scott et al., 1989)
1992	Ablation of *Prnp* by gene targeting in mice (Büeler et al., 1992)
1993	$Prnp^{o/o}$ mice are resistant to scrapie inoculation (Büeler et al., 1993; Sailer et al., 1994)
	Structural differences between PrP^{C} and PrP^{Sc} isoforms noted (Pan et al., 1993)
1994	Cell-free conversion of PrP^{C} to protease-resistant PrP (Kocisko et al., 1994)
1996	New variant of CJD identified (Will et al., 1996)
	BSE prion strain carries a distinct glycotype signature (Collinge et al., 1996)
	First NMR structure of core murine PrP^{C} solved (Riek et al., 1996)
1997	Evidence that nvCJD is caused by the BSE agent (Bruce et al., 1997; Hill et al., 1997a)
	B lymphocytes necessary for peripheral prion pathogenesis (Klein et al., 1997)
1998	Genes controlling incubation period are congruent with *Prnp* (Moore et al., 1998)
1999	Discovery of the PrP^{C} homolog (Moore et al., 1999)
2000	Temporary depletion of lymphoid FDCs impairs prion replication (Montrasio et al., 2000)
	Experimental transmission of BSE in sheep by blood transfusion (Houston et al., 2000)
2001	Complement involved in prion pathogenesis (Klein et al., 2001; Mabbott et al., 2001)
2003	Transgenic expression of soluble PrP inhibits prion replication (Meier et al., 2003)

fied PrP^{Sc} as the first reliable surrogate marker of prion infection. The impact of this technology was phenomenal: even now, twenty years after its original description, the detection of PK-resistant prion protein (termed PrP^{27-30} because of its molecular weight after hydrolysis of its PK-sensitive amino-terminal domain) remains the gold standard for biochemical diagnosis of prion diseases and forms the basis for all of the currently marketed BSE tests (Figure 1).

The second paper, to which one of us had the privilege of contributing, verifies a crucial prediction of Prusiner's protein-only hypothesis (Büeler et al., 1993). If PrP^{Sc} multiplies by imparting its conformation onto host-borne PrP^{C}, organisms devoid of PrP^{C} should be resistant to prion infection. This idea was compelling, but in the early days of prion research, no technology was available that would allow for the targeted removal of a specific gene from the mammalian genome. As soon as in vivo gene ablation became feasible (Zijlstra et al., 1990), Hansrüedi Büeler and Charles Weissmann set out to ablate the *Prnp* gene, which encodes PrP^{C}. $Prnp^{o/o}$ mice were alive and well (Büeler et al., 1992), nowithstanding some minor abnormalities (Collinge et al., 1994; Tobler et al., 1996; Watarai et al., 2003)—some of which may not even be causally related to the prion gene (Aguzzi and Hardt, 2003). The excitement in Zurich was considerable as it became gradually clear that inoculation of $Prnp^{o/o}$ mice with brain homogenate from scrapie-sick mice failed to induce disease of any kind (Büeler et al., 1993) or elicit any subclinical replication of the agent (Sailer et al., 1994).

The study of Büeler and colleagues has sometimes been invoked as the "final proof" of the protein-only hypothesis. That is certainly not the case: the knockout experiment was designed to *disprove* Prusiner's hypothesis—and it would have certainly done so if $Prnp^{o/o}$ mice had developed disease. As always with negative results, alternative interpretations can be offered (Popper, 1991). Those skeptical of the prion hypothesis were quick in pointing out that PrP^{C} may be a receptor for a hitherto unidentified virus, whose ablation would confer antiviral resistance. Yet it is fair to say that the resistance to scrapie of *Prnp* knockout mice constitutes one of the most stringent challenges to the protein-only hypothesis. Hence its failure is very significant.

The availability of $Prnp^{o/o}$ mice has triggered a cascade of technological and conceptual advances. For example, it emerged that PrP^{C}, besides controlling prion replication, is necessary for neuronal damage: $Prnp^{o/o}$ neurons adjacent to infected $Prnp^{+/+}$ brain grafts do not

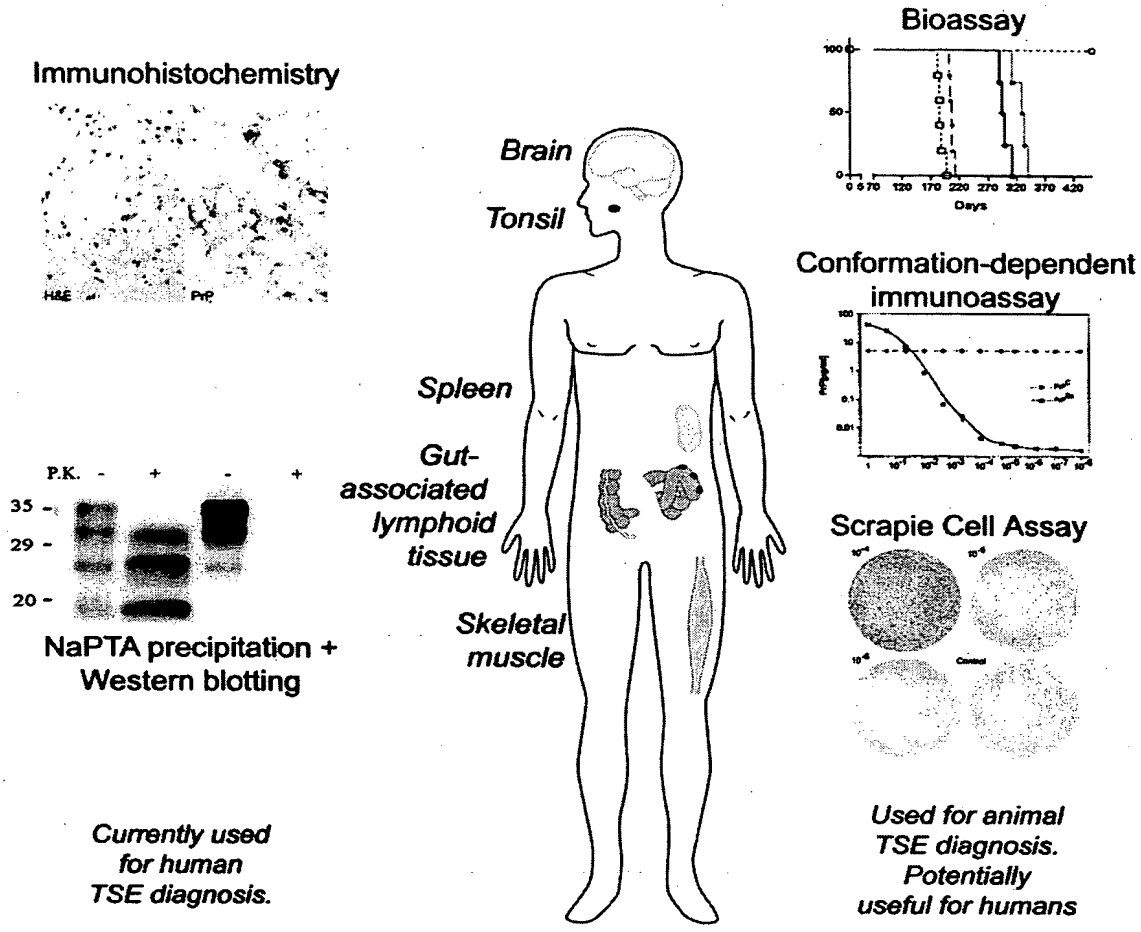


Figure 1. Diagnostic Procedures for Prion Diseases

Synopsis of current diagnostic methods for TSE in humans (left panel) and in experimental animals (right panel). Most methods rely upon the detection of PK-resistant PrP^{Sc}. The tissues in which PrP^{Sc} has been detected in humans are listed in the middle panel. While PrP^{Sc} deposits are most abundant in the CNS, the list of peripheral organs in which PrP^{Sc} can be detected has significantly grown in recent years; it now includes most lymphoid organs as well as skeletal muscle.

incur damage (Brandner et al., 1996a). PrP^{C} is also involved in the transport of the infectious agent from peripheral sites to the central nervous system: its expression appears to be needed in a sessile compartment (Blättler et al., 1997), which is likely to be congruent with stromal components of the lymphoreticular tissue (Montrasio et al., 2000) and of the peripheral nervous system (Glatzel et al., 2001). The microenvironment of lymphoid organs appears to control the velocity of neuroinvasion (Prinz et al., 2003a).

PrP^{C} is not only produced by neurons; its expression is in fact quite ubiquitous, notably including lymphocytes (Cashman et al., 1990) and stromal cells of lymphoid organs (Kitamoto et al., 1991). As a result, wild-type mice enjoy an extremely tight immunological tolerance against PrP^{C}, which had rendered the production of high-affinity immunoreagents very difficult. Instead, the immunization of *Prnp*$^{o/o}$ mice yielded large numbers of very high-affinity antibodies, some of which form the basis for the current crop of BSE tests.

Still, it proved difficult to generate conformational antibodies discriminating between PrP^{C} and PrP^{Sc}. This is surprising in view of the dramatic structural differences between these two isoforms and their differential binding to serum proteins (Fischer et al., 2000). Does the failure of the immune system to generate antibodies specific for PrP^{Sc} indicate that all relevant neoepitopes of PrP^{Sc} that are newly exposed by the conversion of the protein to its disease-associated state are inaccessible? Early claims of discriminatory antibodies, such as Prionics' 15B3 clone (Korth et al., 1997), have not lived up to the expectations. A recently developed antibody against a characteristic tripeptide (YYR) exposed in PrP^{Sc}, but not in PrP^{C}, may be more promising (Paramithiotis et al., 2003). However, the YYR motif is certainly not specific to PrP^{Sc}, and the usefulness of this antibody awaits independent confirmation.

The Spontaneous Generation of Prions

A mesmerizing implication of the protein-only hypothesis is the propagation of prions in an entirely synthetic system. If the infectious agent is a misfolded form of PrP, and its replication is promoted by its interaction

with PrP^C, then the entire process should be, in principle, reproducible in a cell-free environment consisting exclusively of PrP^C, PrP^{Sc}, and maybe some "promoting factors." The importance of such an experiment is immediately evident: de novo generation or amplification of prions from defined components would prove the protein-only hypothesis and set to rest all other alternative explanations (Aguzzi and Weissmann, 1997). Besides, such a system would be extremely valuable for studying the conversion process, for exploring the species barrier phenomenon, and for testing conversion antagonists that may provide therapeutic compounds.

A decisive milestone toward this goal was accomplished with the establishment of an in vitro conversion system based on the coincubation of substantially purified constituents (Kocisko et al., 1994). This seminal work showed that incubation of radiolabeled PrP^C with cold PrP^{Sc} leads to the formation of PK-resistant radiolabeled PrP—indicating that PrP^{Sc} had somehow imparted some of its properties onto PrP^C. The original system required vastly superstochiometric amounts of PrP^{Sc}, which precluded the detection of any increase in prion infectivity. However, the method was used to probe the conversion efficiency between PrP molecules with different primary sequences and thereby, to some extent, the tightness of species barriers (Bessen et al., 1995; Horiuchi et al., 2000). In the intervening years, the in vitro conversion methodology has yielded remarkable insights and even assays for identification of antiprion compounds.

It was reported that PrP^{Sc} could be amplified by cycles of sonication followed by incubation with brain homogenate (Saborio et al., 2001). The idea behind this experiment was that sonication might fracture large PrP^{Sc} aggregates into smaller units, each one of which would accrue PrP^C and act as independent "infectious unit." Several skeptics, however, have pointed out that this intriguing report is still awaiting independent confirmation. Also, more than two years after its publication, no evidence has come forward that this "protein misfolding cyclic amplification" would augment the infectivity of any given sample.

Along parallel lines, conditions were established at that recombinantly produced PrP was transformed into an isoform termed βPrP, with several typical properties of PrP^{Sc} (Jackson et al., 1999): increased β sheet content, aggregability, and resistance to PK. This molecule was deemed quite interesting for two main reasons. Firstly, one had hoped that immunization of mice with βPrP might give rise to conformation-specific monoclonal antibodies, which would help in discriminating directly between PrP^C and PrP^{Sc}. The latter would render obsolete the venerable PK digestion assay and may facilitate the development of higher-throughput PrP^{Sc} immunoassays. Secondly, βPrP might be equivalent to PrP^* (Weissmann, 1991), a metastable intermediate postulated to arise during conversion of PrP^C to PrP^{Sc}. If so, inoculation of mice with suitable amounts of βPrP might result in the generation of transmissible disease.

At present βPrP has yet to fulfill either of these two expectations. Yet it is not implausible that additional experimentation in specifically devised animal models may change this negative outcome. As with transmission of kuru to chimps, it is advisable to be patient.

Form Follows Function

If the protein-only hypothesis is correct, one could argue that the prion problem is, in essence, one of protein structure. Whether prions multiply by template-directed refolding or by seeded nucleation, certain domains of PrP^C (or the entire protein) would need to rearrange such that the monomeric protein becomes capable of inducing the same change in further PrP^C monomers (Figure 2A). This idea represents the core of the "template-directed refolding" hypothesis, which predicates an instructionist role for PrP^{Sc} onto PrP^C. The experimental evidence is compatible with this hypothesis, yet no positive evidence in its favor has come forward.

Alternatively, it has been proposed that PrP^{Sc} exists in a mass-action equilibrium with PrP^C. Such equilibrium would be heavily shifted toward the side of PrP^C so that only minute amounts of PrP^{Sc} would coexist with PrP^C. If that were the case, PrP^{Sc} could not possibly represent the infectious agent since it would be ubiquitous. According to this "nucleation" hypothesis (Jarrett and Lansbury, 1993), however, the infectious agent would consist of a highly ordered aggregate of PrP^{Sc} molecules. The aggregated state would be an intrinsic property of infectivity: monomeric PrP^{Sc} would be harmless, but it might be prone to incorporation into nascent PrP^{Sc} aggregates (Figure 2B).

Testing these hypotheses requires precise knowledge of the structural features of both PrP^C and PrP^{Sc}. To date, such knowledge has not progressed to a state that would allow for resolution of this question. The structure of PrP^C has been studied extensively with high-resolution methods. Both crystallography (Knaus et al., 2001) and nuclear magnetic resonance (NMR) spectroscopy (Riek et al., 1996) have yielded detailed insights into the arrangement of PrP^C at the atomic level. PrP^{Sc}, however, has been amenable merely to low-resolution structural methods.

The NMR studies of recombinant PrP^C yielded a big surprise. The amino-proximal half of the molecule is not structured at all, whereas the carboxy-proximal half is globular and contains three α helices (Riek et al., 1996, 1997). This does not mean that the amino terminus must be randomly coiled in vivo: functional studies in transgenic mice imply that the domain comprising amino acids 32-121 carries out important physiological functions (Shmerling et al., 1998). Maybe the flexible tail of PrP^C acquires a defined structure once it reaches its natural habitat on rafts, which are specialized microdomains of the plasma membrane (Naslavsky et al., 1997).

Why wasn't it yet possible to elucidate the structure of PrP^{Sc}? As discussed above, prion infectivity can be recovered only from prion-infected mammalian organisms or (in much lesser quantities) from infected cultured cells. In neither case is the purity of the recovered material satisfactory. Moreover, infectivity-associated PrP^{Sc} appears to consist obligatorily of aggregates; disaggregation sterilizes prions (Prusiner et al., 1981). But insoluble aggregates are resilient to most technologies for determination of protein structure; hence all we know is that PrP^{Sc} consists mainly of β-pleated sheet (Caughey et al., 1991) and that PrP^{Sc} aggregates expose a remarkably ordered structure (Wille et al., 2002).

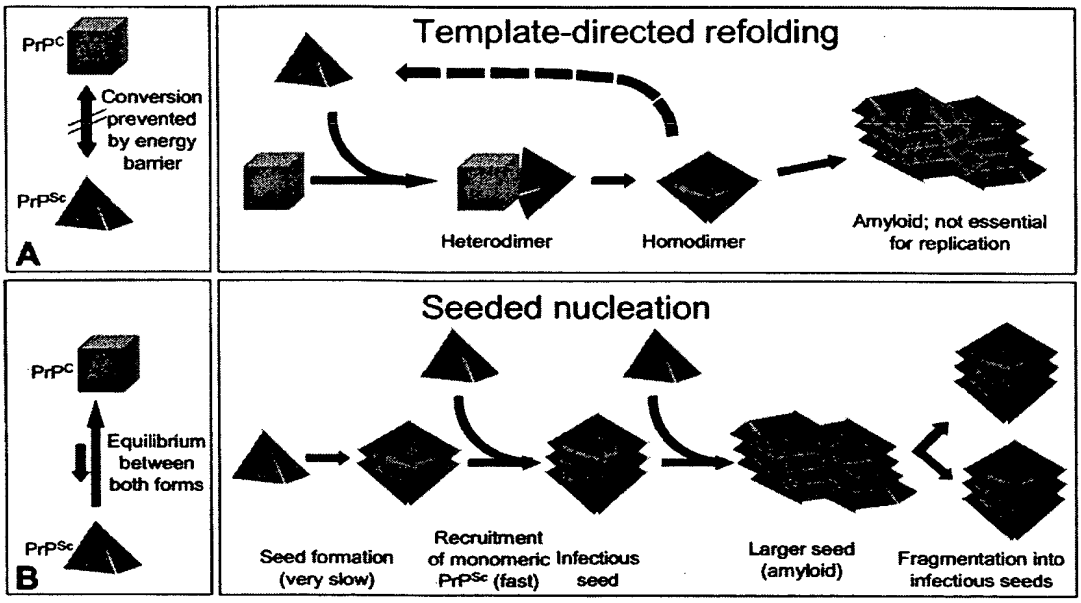


Figure 2. Models for the Conformational Conversion of PrP^C into PrP^{Sc}

(A) The "refolding" or template assistance model postulates an interaction between exogenously introduced PrP^{Sc} and endogenous PrP^C, which is induced to transform itself into further PrP^{Sc}. A high energy barrier may prevent spontaneous conversion of PrP^C into PrP^{Sc}.

(B) The "seeding" or nucleation-polymerization model proposes that PrP^C and PrP^{Sc} are in a reversible thermodynamic equilibrium. Only if several monomeric PrP^{Sc} molecules are mounted into a highly ordered seed, further monomeric PrP^{Sc} can be recruited and eventually aggregates to amyloid. Within such a crystal-like seed, PrP^{Sc} becomes stabilized. Fragmentation of PrP^{Sc} aggregates increases the number of nuclei, which can recruit further PrP^{Sc} and thus results in apparent replication of the agent.

Yeast Prions

Thirty years ago, Francois Lacroute described mysterious yeast traits that apparently propagated by nonmendelian genetics (Lacroute, 1971). For two decades, this phenomenon remained unexplained—until Reed Wickner proposed that the unusual genetic properties of these mutants could be explained by a prion-like behavior of two previously identified yeast proteins: Sup35p, an essential component of the translation termination machinery, and Ure2p, a protein that regulates nitrogen metabolism (Wickner, 1994). Further yeast prions were identified in the following, so that one could now argue that the yeast prion phenomenon is much better understood than its mammalian counterpart. The prion-forming domain (PrD) of Sup35p is modular and transferable; artificial prions were generated by fusing a mammalian receptor to the Sup35p PrD (Li and Lindquist, 2000).

In the prion-infected state (termed ψ^+), Sup35p is sequestered into fibrils. As consequence, termination of translation is impaired, and reading frames situated downstream of nonsense codons can be translated into proteins (Figure 3). Just like in street traffic, ignoring stop signs does not generally constitute healthy behavior, but Susan Lindquist made a convincing case that such transgressions may play a decisive role in creating "evolutionary buffers." By occasionally switching on bicistronic reading frames through the ψ^+ state, yeast cells can reversibly probe the effects of combinatorial expression of mutated genes, hence creating additional layers of evolutionary variation (True and Lindquist, 2000).

BSE and Other Prion Threats to Humans

When Stanley Prusiner started his first attempts at tackling the problem of TSE (Prusiner et al., 1977), this group of diseases was not exactly in the public limelight. However, bovine spongiform encephalopathy (BSE) was recognized a few years later (Wells et al., 1987) —an event that would dramatically change the public perception of prion diseases. CJD was, and fortunately continues to be, exceedingly rare: its incidence is typically $1/10^6$ inhabitants/year, but reaches $3/10^6$ inhabitants/year in Switzerland, which is currently reporting the highest number of cases (Glatzel et al., 2002, 2003b). Kuru, once decimating the population of Papua New Guinea, has almost disappeared. Iatrogenic transmission of CJD has principally occurred through improperly sterilized neurosurgical instruments, transplants of dura mater, and administration of pituitary hormones of cadaveric origin. While the two latter routes of transmission no longer pose a major threat, a significant number of individuals may have been infected during a critical time window and may develop CJD in the coming years.

Variant CJD (vCJD) has caused some 140 deaths in the United Kingdom and a few cases in France, Italy, and Canada (http://www.doh.gov.uk/cjd/stats/aug02.htm). Epidemiological, biochemical, and histological evidence suggests that vCJD represents transmission of bovine spongiform encephalopathy (BSE) prions to humans (Aguzzi, 1996; Aguzzi and Weissmann, 1996; Bruce et al., 1997; Hill et al., 1997a). The incidence of vCJD in the United Kingdom rose each year from 1996 to 2001, evoking fears of a large upcoming epidemic.

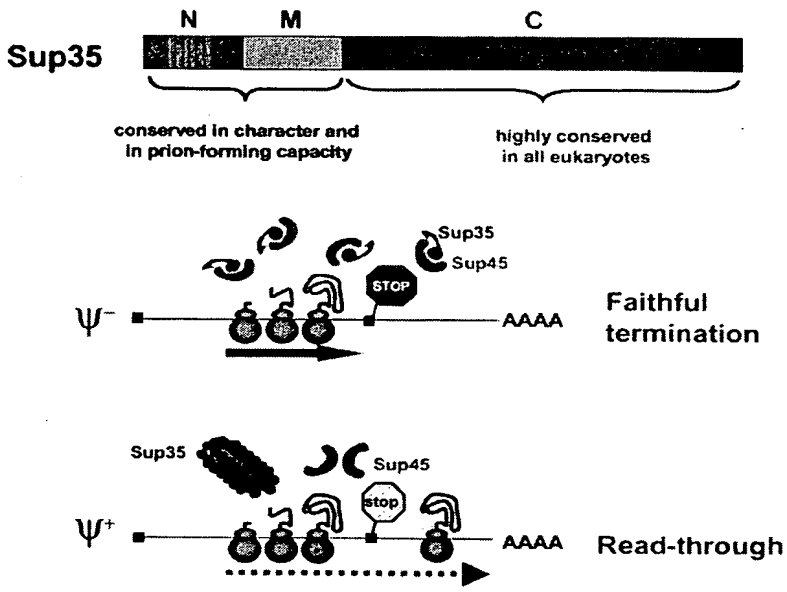


Figure 3. Function of the Yeast Prion, Sup35

(A) Sup35 consists of an amino-terminal glutamine-rich module crucial for conversion into the prion state.

(B) In the ψ^- state, Sup35 is required for reliable termination of translation.

(C) In ψ^+ yeast cells, however, Sup35 is sequestered in ordered fibrillary aggregates. Shortage of functional Sup35 leads to transgressions in stop codon recognition and translation of downstream reading frames (red line). In the off state, such pseudogenes may accumulate otherwise toxic mutations. Acquisition of the on (ψ^+) state may lead to the appearance of new phenotypes, hence increasing the complexity of genetic variability.

Since the year 2001, however, the incidence of vCJD in the UK appears to be stabilizing (http://www.cjd.ed.ac.uk/vcjdq.htm). One may argue that it is too early to draw any far-reaching conclusions, but each year passing without any dramatic rise in the number of cases increases the hope that the total number of vCJD victims will be limited (Valleron et al., 2001). Presently, there is reason to hope that the incidence of vCJD in the United Kingdom may already be subsiding (Andrews et al., 2003).

vCJD prions accumulate prominently in lymphoreticular tissue, and the latter can be used for diagnostic purposes. Surprisingly, prions accumulate in lymphoid organs and muscle of sporadic CJD patients (Glatzel et al., 2003a).

There is uncertainty surrounding the danger of transmission to humans represented by chronic wasting disease. In fact, even transmissibility of BSE to humans relies on circumstantial evidence. Epidemiology and biochemistry favor the link between BSE and vCJD, but are not ultimately conclusive. The Koch postulates (which would unambiguously assign an infectious agent to a disease) have never been fulfilled, and experimental inoculation of humans was fortunately never performed. Also, accidental exposure to BSE infectivity of a sizable collective at a precisely defined time point has never occurred, or did not result in disease. Likewise, we do not know whether scrapie is just a veterinarian problem that affects only sheep and goat or whether it can cross species barriers and affect humans. Finally, it is unknown whether BSE, upon transmission to sheep, remains as dangerous for humans as cow-derived BSE, or whether it becomes attenuated and acquires the (allegedly) innocuous properties of bona fide sheep scrapie.

The Elusive Function of PrP^C

In spite of the fact that the first *Prnp* knockout mice are available since 12 years (Büeler et al., 1992), the normal function of the cellular prion protein is still unknown. A number of subtle abnormalities have been described in PrP-deficient mice (Collinge et al., 1994; Tobler et al., 1996), but their molecular basis is undefined, and there may be some variability due to the genetic background of the mice utilized. Hence, the only definite phenotype of $Prnp^{o/o}$ mice is their resistance to prion inoculation (Büeler et al., 1993) —yet it seems unlikely that a singular protein that is as highly conserved among species as PrP^C, from turtles to frogs, fish, and humans, has evolved for the sole reason of bestowing susceptibility to prion diseases.

If the function of PrP^C were completely unrelated to prion disease pathogenesis, one might argue that PrP^C is just one of many thousands proteins whose function awaits clarification—but why should then the elucidation of the function of PrP^C be given any priority? On the other hand, the function of PrP^C may very well have something to do, in a subtle way, with prion-induced damage. *Prnp* ablation does not elicit disease, even when induced postnatally (Mallucci et al., 2002); hence prion pathology is unlikely to come about by loss of PrP^C function. But assume that PrP^C transduces a signal, or that it possesses some enzymatic activity. If so, conversion to PrP^{Sc} may alter signal transduction strength, or substrate specificity, thereby conferring a toxic dominant function. In these scenarios, understanding the function of PrP^C may help in deciphering prion pathology and maybe even devising therapeutical approaches.

So, what is the evidence that PrP^C may be a signal transducer or an enzyme? Speculations on both hypotheses abound, but facts are scarce. Crosslinking PrP^C with $F(ab)_2$ antibody fragments has been reported to activate intracellular tyrosine kinases (Mouillet-Richard et al., 2000). However, this phenomenon was not reported to occur in vivo, and the only cell line in which it was described was never made available to the scientific community for independent verification. This does not exclude that PrP^C functions as a signal transducer, but the present case is of limited strength.

Is PrP^C an enzyme? Glockshuber noted that PrP^C has similarities to membrane-anchored signal peptidases (Glockshuber et al., 1998), but his observation has not been substantiated by functional data. The speculation that PrP^C may be a superoxide dismutase (Brown et al., 1997, 1999) was perceived as particularly attractive in view of its multiple copper binding sites, and it was recently suggested that amino-proximally truncated PrP^C may depress endogenous dismutase activity (Sakudo et al., 2003). However, PrP^C does not make any measurable contribution to dismutase activity in vivo (Hutter et al., 2003; Waggoner et al., 2000).

Maybe PrP^C and PrP^{Sc} do not possess any intrinsic biological activity, yet they modify the function of other proteins. This supposition has prompted a search for PrP-interacting partners, and there is no dearth of PrP binding proteins: the antiapoptotic protein Bcl-2 (Kurschner et al., 1995), caveolin (Gorodinsky and Harris, 1995; Harmey et al., 1995), the laminin receptor precursor (Rieger et al., 1997), plasminogen (Fischer et al., 2000), and N-CAM (Schmitt-Ulms et al., 2001). None of these interactors, however, have yet revealed a functional pathway in which PrP^C would be involved in vivo. It was recently shown that PrP-deficient macrophages do not support bacterial "swimming internalization" of the Gram-negative bacterium, *Brucella abortus* (Watarai et al., 2003), and that PrP^C interacts with a *Brucella* heat shock protein, Hsp60. These findings raise the question of whether PrP^C may participate in a general Hsp60-dependent "danger sensing" mechanism (Aguzzi and Hardt, 2003).

A Doppelganger of the Prion Protein

The original $Prnp^{o/o}$ mice did not display any severe abnormalities. However, some of the knockout lines generated later, i.e., Ngsk $Prnp^{-/-}$ (Sakaguchi et al., 1996), ZH-II $Prnp^{-/-}$ (Rossi et al., 2001), and Rcm0 mice (Moore et al., 1999), develop progressive cerebellar Purkinje cell degeneration with ataxia in advanced age. This phenotype was originally attributed to the lack of PrP^C and ran counter to the two PrP knockout mouse lines produced earlier: the ZH-I $Prnp^{o/o}$ (Büeler et al., 1992) and the Edbg $Prnp^{-/-}$ mice (Manson et al., 1994). The characterization of Ngsk $Prnp^{-/-}$ mice was particularly conscientious: the authors reintroduced *Prnp* as a transgene by genetic crosses and showed that this manipulation rescued the Purkinje cell degeneration. It seemed entirely reasonable, hence, to conclude that PrP^C is necessary for cerebellar homeostasis. Yet this interpretation could not be easily reconciled with the lack of phenotype in the remaining knockout lines and eventually was proven to be incorrect.

The inconsistency was eventually resolved by David Westaway's discovery of a novel gene located just 16 kilobases downstream of *Prnp* and encoding a 179 residue protein that has sequence similarities to the C terminus of PrP and was thus termed Doppel or Dpl (Moore et al., 1999). It then emerged that the gene targeting strategy in all ataxic PrP-deficient mice was associated with deletion of a splice acceptor site located on the coding exon of *Prnp*. This modification effectively places Dpl under transcriptional control of the *Prnp* promoter. As a consequence, brain expression of Dpl, which is normally very low, skyrockets in Nsgk, ZH-II, and Rcm0 mice (Weissmann and Aguzzi, 1999). This is clearly neurotoxic, as ablation of the Dpl reading frame from ZH-II mice abolishes the Purkinje cell degeneration phenotype (Nicolas Genoud, Axel Behrens, and A.A., unpublished data).

Most intriguingly, Dpl-dependent neurodegeneration is abolished by cell-autonomous coexpression of full-length PrP (Rossi et al., 2001). Formally, this indicates that Dpl and PrP^C act antagonistically, maybe because they bind to a hitherto conjectural common ligand (Figure 4A), which was provisionally termed L_{PrP} (Shmerling et al., 1998). Alternatively, PrP^C and Dpl might engage in heterooligomeric complexes (Figure 4B), whose function could depend on their stoichiometric composition (Behrens and Aguzzi, 2002). The same mechanism may be operative in transgenic mice produced by Doron Shmerling and Charles Weissmann (Shmerling et al., 1998) in an attempt to specify the domain of PrP^C required for prion replication. Expression of a PrP variant that lacks a large part of the N terminus of PrP in $Prnp^{o/o}$ mice induces spontaneous cerebellar degeneration, which however affects granule cells rather than Purkinje cells (the promoter used was inactive in Purkinje cells) and can also be prevented by the coexpression of a single endogenous *Prnp* allele. Structural studies have shown that human Dpl contains a relatively short, flexibly disordered "tail" comprising residues 24-51 and a globular domain extending from residues 52 to 149 for which a detailed structure was obtained (Luhrs et al., 2003). Despite their highly divergent primary sequence, Dpl is largely superimposable to the carboxy-proximal half of PrP^C.

The molecular pathways by which Dpl and amino-proximally truncated PrP damage the cerebellum are unknown. However, the suppressibility of both phenotypes by full-length PrP^C is indicative of a high degree of specificity. Therefore, we contend that this model presently represents the best validated window of entry to determine the function of PrP^C in vivo.

The Basis of Prion Neurotoxicity

PrP^{Sc} accumulation in the brain is the hallmark of prion diseases, and PrP^{Sc} is—for all we know—a major component of the infectious agent. But is PrP^{Sc} also directly responsible for the devastating CNS pathology typical of prion diseases? On the one hand, accumulation of amyloid (or preamyloid) in the CNS is likely to be generally unhealthy, as exemplified by Alzheimer's disease (Aguzzi and Haass, 2003) and cerebral vascular amyloidoses (Revesz et al., 2002). On the other hand, chronic deposition of PrP^{Sc} does not damage *Prnp* knockout brains (Brandner et al., 1996a), and depletion of PrP^C from neurons of scrapie-infected mice prevents disease (Mallucci et al., 2003). Therefore, accumulation of PrP^{Sc} is unlikely to fully account for prion pathology. If so, what is it that actually kills the neurons?

Brains of Creutzfeldt-Jakob disease victims look truly frightening. In heavily affected areas, there is hardly any neuron left, and the brain tissue texture is coarsened by the abnormal growth of astrocytes ("gliosis") and microglial cells. The most telling hallmark is spongiosis, a peculiar microvacuolation affecting residual neural cells.

The molecular steps that emanate from prion replication and lead to such destruction are unknown. Some

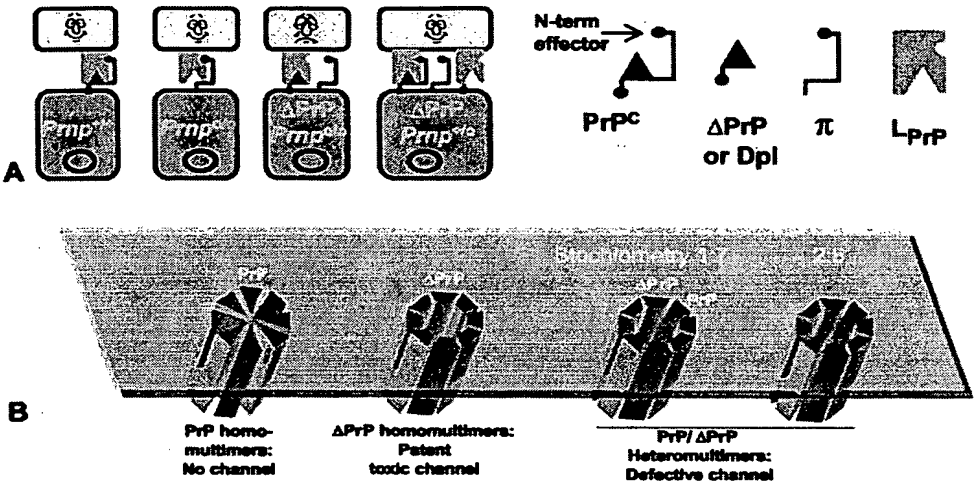


Figure 4. Hypothetical Models for the Function of PrP^C and the Neurotoxicity of ΔPrP^C and Dpl

(A) PrP^C and Dpl (or ΔPrP) may compete for a common ligand, provisionally termed L_{PrP}. In order to accommodate the lack of neurodegeneration in *Prnp*$^{0/0}$ mice, however, one would have to postulate the existence of a functional PrP^C analog, here termed π. While this model accommodates all experimental findings known to date, no physical evidence has come forward for the existence of L_{PrP} and π.

(B) Dpl and ΔPrP may form a homomultimeric toxic aggregate, which may be inactivated by participation of full-length PrP^C. Toxicity may come about by various hypothetical mechanisms. For example, if such aggregates were to span a membrane, toxic properties may relate to the formation of pores.

gain of toxic function is likely, as constitutive or postnatal depletion of PrP^C does not trigger any pathology. A lively discussion is developing on the role of abnormal PrP^C topologies. Targeting of PrP to the cytosol results in rapidly lethal neurodegeneration (yet without PrP^{Sc}), and proteasome inhibition induces a slightly protease-resistant PrP species in cultured cells, which may be self-sustaining—at least for a while (Ma and Lindquist, 2002; Ma et al., 2002). Therefore, prion toxicity may start with retrotranslocation of PrP^C from the endoplasmic reticulum to the cytosol, in conjunction with impaired proteasomal function. While PrP is clearly toxic in the cytosol, the details of how it may get there are debated. Cytosolic PrP retains its secretory leader peptide and does not contain a glycosyl phosphatidyl inositol anchor, suggesting that it never enters the endoplasmic reticulum (Drisaldi et al., 2003). Whether toxicity of cytosolic PrP is universal, however, is currently quite hotly discussed (Roucou et al., 2003). On the other hand, Lingappa found that PrP^C assumes a transmembrane topology (^{Ctm}PrP), whose concentration correlates with neurotoxicity (Hegde et al., 1998, 1999). These data suggest that ^{Ctm}PrP represents a major toxic moiety.

We still know nothing of the biochemical pathways leading to brain damage, be they triggered by cytoplasmic PrP or by ^{Ctm}PrP; these may lead to the identification of therapeutic targets and may share components with other neurodegenerative diseases.

The Future of Prion Therapeutics

An impressive wealth of molecules was touted as potential antiprion lead compounds. However, none of these therapeutical leads have proven their usefulness yet in clinical settings, and some have conspicuously failed. One of the possible problems derives from the fact that most antiprion compounds were identified in cell culture assays, where chronically prion-infected neuroblastoma cells are "cured" of their PrP^{Sc} and prion burden. A startling variety of substances appears to possess such prion-curing properties; a nonexhaustive list includes compounds as diverse as Congo red (Caughey and Race, 1992), amphotericin B, anthracyclins (Tagliavini et al., 1997), sulfated polyanions (Caughey and Raymond, 1993), porphyrins (Priola et al., 2000), branched polyamines (Supattapone et al., 2001), "β sheet breakers" (Soto et al., 2000), and the spice curcumin (Caughey et al., 2003).

Disappointingly, none of these compounds proved very effective for actual therapy of sick animals—let alone patients. We therefore believe that it is premature to treat patients with alleged antiprion drugs on the sole basis of antiprion efficacy in neuroblastoma cells. This shortcut was taken in the case of quinacrine, which cures scrapie-infected cultured cells with impressive efficacy (Korth et al., 2001), yet appears to be utterly ineffective in scrapie-infected mice (Collins et al., 2002) and in CJD patients (Cooper, 2002), besides being severely hepatotoxic (Scoazec et al., 2003).

Why do scrapie-infected cells fare so poorly as a model system for prion therapy? In our experience, infection rarely hits all cells in any given culture, and the prion-infected state can be quite unstable. Therefore, one could speculate that a variety of stressors may masquerade as antiprion cures by conferring a selective advantage to noninfected cells. This interpretation would explain the puzzling observation that antiprion "cure" is brought about by compounds with no structural or biological similarities.

Cytidyl-guanyl oligodeoxynucleotides (CpG-ODN), which bind Toll-like receptor 9 (TLR9) and stimulate innate

immune responses, were reported to delay disease upon chronic administration to scrapie-infected mice (Sethi et al., 2002). The contention that immune stimulation might protect against prions is extraordinary and is difficult to reconcile with the observation that immune deficiencies of all kinds inhibit prion spread (Frigg et al., 1999; Klein et al., 1997, 1998, 2001; Prinz et al., 2003c). Besides, MyD88$^{-/-}$ mice undergo normal prion pathogenesis despite abrogation of TLR9 signaling (Prinz et al., 2003b), and we could not evidence any major effects of TLR9 stimulation on the course of disease—in a paradigm identical to that described originally (M.P., M. Heikenwälder, and A.A., unpublished data). Instead, repeated CpG-ODN administration proved extremely lymphotoxic (Heikenwalder et al., 2004)—a fact that may well explain its antiprion properties.

On a more positive note, the tremendous interest in this field has attracted researchers from various neighboring disciplines, including immunology, genetics, and pharmacology, and therefore it is to hope that rational and efficient methods for managing prion infections will be developed in the future.

Immunotherapy against Prions?

Prions are sturdy and their resistance against sterilization is proverbial, yet exposure in vitro to anti-PrP antisera can reduce the titer of infectious hamster brain homogenates (Gabizon et al., 1988). Anti-PrP antibodies were found to inhibit formation of protease-resistant PrP in a cell-free system (Horiuchi and Caughey, 1999). Also, antibodies (Klein et al., 2001) and F(ab) fragments to PrP (Enari et al., 2001; Peretz et al., 2001) can suppress prion replication in cultured cells.

While these data suggest the feasibility of antiprion immunoprophylaxis, the mammalian immune system is essentially tolerant to PrP^{C}; this is hardly a surprise, given that PrP^{C} is expressed on T and B cells. Ablation of *Prnp* (Büeler et al., 1992) renders mice highly susceptible to immunization with prions (Brandner et al., 1996b), and indeed some of the best monoclonal antibodies to PrP^{C} were generated in *Prnp*$^{o/o}$ mice (Prusiner et al., 1993).

Tolerance was circumvented by transgenic expression of an immunoglobulin μ chain containing the epitope-interacting region of 6H4, a high-affinity anti-PrP monoclonal antibody (Korth et al., 1997). The transgenic μ chain associated with endogenous κ and λ chains, some pairings lead to reactive moieties and, consequently, to high anti-PrP^{C} titers in *Prnp*$^{o/o}$ and *Prnp*$^{+/+}$ mice. The buildup of anti-PrP^{C} titers, however, was more sluggish in the presence of endogenous PrP^{C}, suggesting that clonal deletion is actually occurring. B cell clones with the highest affinity to PrP^{C} are probably eliminated by tolerance, while clones with medium affinity are retained (Figure 5A). The latter sufficed to block prion pathogenesis upon intraperitoneal prion inoculation (Heppner et al., 2001). Hence, B cells are not intrinsically tolerant to PrP^{C} and can, in principle, mount a protective humoral response against prions. It was then found, in a followup study, that passive transfer of anti-PrP monoclonal antibodies (in admittedly heroic amounts) can delay the onset of scrapie in mice infected with prions intraperitoneally, albeit not such infected intracerebrally (White et al., 2003).

The challenges to a practical antiprion immunization, however, are enormous. While providing an encouraging proof of principle, transgenic immunization cannot easily be reduced to practice. Further, no protection was observed if treatment was started after the onset of clinical symptoms, suggesting that passive immunization might be a good candidate for prophylaxis rather than therapy of TSEs. Active immunization, like in most antiviral vaccines, may be more effective, but is rendered exceedingly difficult by the stringent tolerance to PrP^{C} (Souan et al., 2001; F. Heppner, E. Pellicioli, M.P., and A.A., unpublished results; and Figure 5B).

Soluble Prion Antagonists

In several paradigms, expression of two PrP^{C} moieties subtly different from each other antagonizes prion replication. For example, humans heterozygous for a common *Prnp* polymorphism at codon 129 are largely protected from CJD: this effect is so important that it may have acted as selective evolutionary pressure (Mead et al., 2003). Similarly, transgenic expression of hamster PrP^{C} renders *Prnp*$^{o/o}$ mice highly susceptible to hamster prions, whereas coexpression of mouse PrP^{C} diminishes this effect. Transdominant single nucleotide mutations of *Prnp* have also been described (Perrier et al., 2002).

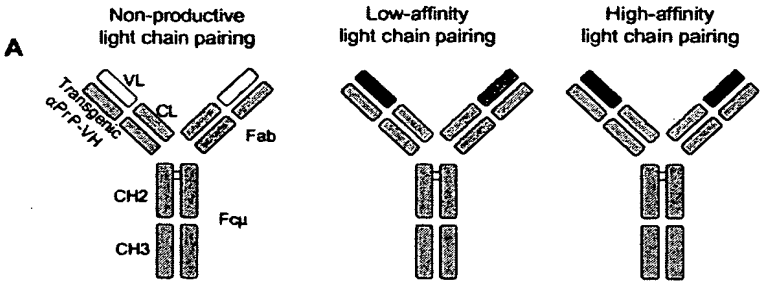
The molecular basis for these effects is unknown; perhaps the subtly modified PrP^{C} acts as a decoy by binding incoming PrP^{Sc} (or protein X) and sequestering it into a complex incapable of further replication.

We tested the latter hypothesis by fusing an immunoglobulin Fcγ domain to PrP^{C}. The Fcγ tail served multiple purposes: (1) ligand dimerization, which may enhance its avidity for interacting partners; (2) provision of a convenient tag for affinity purification; (3) expression of the protein as a soluble moiety, which allows for testing cell-autonomous effects; and (4) increased stability in body fluids. Excitingly, the PrP-Fc$_2$ fusion protein was found to compete with PrP^{C} for PrP^{Sc} (Figure 6) and to prolong the latency period of prion infection upon expression in transgenic mice (Meier et al., 2003). It will be exciting to determine whether PrP-Fc$_2$ can act cell-autonomously when delivered as a drug. If that proves true, soluble prion protein mutants may represent useful prionostatic compounds.

Prion Diagnosis: Weaknesses and Challenges

Like in any other disease, early diagnosis would significantly advance the chances of success of any possible interventional approach. But when compared to other fields of microbiological diagnostics, the tools for prion diagnosis appear to be depressingly unsophisticated. Presymptomatic diagnosis is virtually impossible, and the earliest possible diagnosis is based on clinical signs and symptoms. Hence, prion infection is typically diagnosed after the disease has considerably progressed.

A significant advance in prion diagnostics was accomplished in 1997 by the discovery that protease-resistant PrP^{Sc} can be detected in tonsillar tissue of vCJD patients (Hill et al., 1997b). It was hence proposed that tonsil biopsy may be the method of choice for diagnosis of vCJD (Hill et al., 1999). Furthermore, there have been reports of individual cases showing detection of PrP^{Sc} at preclinical stages of the disease in tonsil (Schreuder et al., 1996) as well as in the appendix (Hilton et al., 1998), indicating that lymphoid tissue biopsy may be useful for



B

Genetic backround	procedure	fate of B-lymphocytes
Prnp$^{o/o}$	VH transgenesis	maturation
Wild-type	VH transgenesis	moderate deletion
PrP overexpressor	VH transgenesis	mainly deletion
Prnp$^{o/o}$	immunization	vigorous response
Wild-type	immunization	no response

Figure 5. Affinity of Antibodies, Tolerance, and Immunity against Prions

(A) When forced to express a transgenic heavy chain with anti-PrP specificity, B lymphocytes may couple it to a large repertoire of endogenous light chains. Some of the VH-VL pairs (variable domains of heavy and light chains) may yield very high-affinity antibodies, whereas others will have low or no affinity. (B) Mendelian crosses of α-PrP-VH transgenic mice with *Prnp*$^{o/o}$, wild-type, and PrP^{C}-overexpressing transgenic mice informed on tolerogenic constraints. In the absence of endogenous PrP^{C}, mouse sera exhibited high anti-PrP^{C} titers. In wild-type mice, anti-PrP^{C} titers despite some clonal deletion, whereas massive overexpression of PrP^{C} led to dramatic lymphopenia (Heppner et al., 2001). Instead, active immunization yields consistently high anti-PrP^{C} titers only in *Prnp*$^{o/o}$ mice. The permissivity of B lymphocytes to expression of anti-PrP^{C} specificities implies that tolerance to PrP^{C} is predominantly dictated by T-helper constraints. CH: Constant region of the heavy chain. Fab: antigen binding fragment. Fcμ: IgM-specific heavy chain.

diagnosing presymptomatic individuals. These observations triggered large screenings of human populations for subclinical vCJD prevalence using appendectomy and tonsillectomy specimens (Glatzel et al., 2003b). PrP^{Sc}-positive lymphoid tissue was long considered to be a vCJD-specific feature that would not apply to any other forms of human prion diseases (Hill et al., 1999). However, a recent survey of peripheral tissues of patients with sporadic CJD has identified PrP^{Sc} in as many as one-third of skeletal muscle and spleen samples

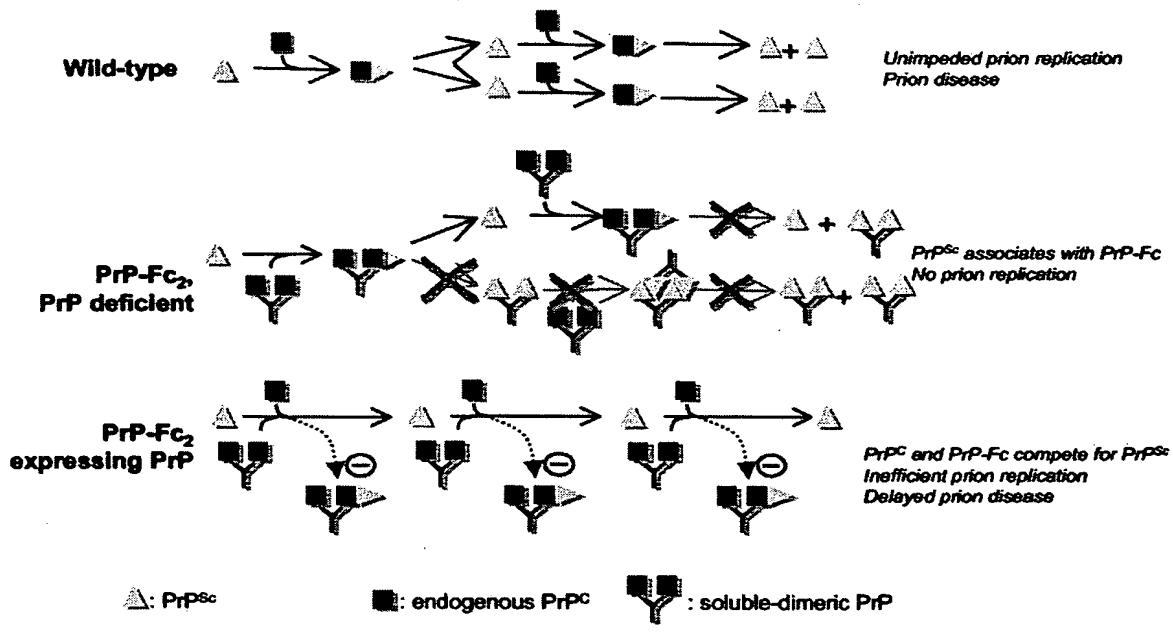


Figure 6. A Model for the Antiprion Action of PrP-Fc$_2$

The template refolding model of prion replication (top) postulates a transient dimerization of PrP^{C} and PrP^{Sc}. As a result, PrP^{Sc} would impart its own β sheet-rich, protease-resistant conformation onto PrP^{C}. In the absence of PrP^{C}, soluble dimeric PrP does not support replication of the infectious agent, nor formation of a protease-resistant moiety (middle). Although several lines of evidence indicate that it can associate with PrP^{Sc}, this association is nonproductive. Mice coexpressing PrP^{C} and soluble dimeric PrP replicate prions and eventually develop scrapie. However, the kinetics with which scrapie pathology develops, prion infectivity replicates, and PrP^{Sc} accumulates is slower than in wild-type mice. All experimental evidence presented here suggests that PrP-Fc$_2$ sequesters incoming as well as nascent PrP^{Sc} and renders it unavailable for further template-directed conversion of PrP^{C} (bottom).

(Glatzel et al., 2003a), as well as the olfactory epithelium of patients suffering from sCJD (Zanusso et al., 2003). These unexpected findings raise the hope that minimally invasive diagnostic procedures may take the place of brain biopsy in intravital CJD diagnostics.

The sensitivity of PrP^{Sc} detection was significantly improved by the sodium phosphotungstic (NaPTA) precipitation method (Safar et al., 1998; Figure 1). By concentrating PrP^{Sc} prior to Western blot analysis, this procedure improves the sensitivity of diagnostic assays by as much as 4 orders of magnitude (Wadsworth et al., 2001). An interesting development was brought about by the conformation-dependent immunoassay (CDI), in which conformational differences of PrP isoforms are mapped by quantitating the relative binding of antibodies to denatured and native protein (Safar et al., 1998). Rather than relying on protease resistance, the CDI measures a variety of misfolded PrP isoforms, which may increase its sensitivity (Bellon et al., 2003; Safar et al., 2002).

Be this as it may, all techniques described above suffer from the fact that PrP^{Sc} continues to represent a surrogate marker for prion infectivity—since (1) PrP^{Sc} has not been incontrovertibly shown to be congruent with the prion, and (2) several manipulations in vitro and in vivo can render PrP^{C} protease resistant without bestowing infectivity on it (Jackson et al., 1999). Therefore, determination of prion infectivity by bioassay remains the golden standard; like in Pasteur's age, the concentration of the infectious agent is determined by inoculating serial dilutions of the test material into experimental animals, and the dilution at which 50% of the animals contract the disease (termed ID_{50}) is determined. Naturally, this system is riddled with inconveniences: scores of animals need to be sacrificed, and the incubation times are lengthy (transgenetic overexpression of PrP^{C} can help, but only to some extent). Also, the method tends to be breathtakingly inaccurate: the inoculation schemes used in most studies typically suffer from standard errors of ± 1 order of magnitude!

A radical improvement of this situation is likely to be brought about by the use of prion-susceptible cell lines (Bosque and Prusiner, 2000; Race et al., 1987). The determination of prion infectivity endpoints in cultures of highly susceptible cells combines the sensitivity and intrinsic biological validity of the bioassay (i.e., direct measurement of the infectivity) with the speed and convenience of an in vitro methodology amenable to medium-throughput automation (Klohn et al., 2003).

Unresolved Problems in Prion Science

The study of prions has taken several unexpected directions over the past few years. However, the areas that are still obscure do not relate only to the details; some of them concern the core of the prion concept (Chesebro, 1998). In summary, there are five large groups of questions regarding the basic science of prion replication and of development of transmissible spongiform encephalopathies diseases:

- Which are the molecular mechanisms of prion replication? How does the disease-associated prion protein, PrP^{Sc}, achieve the conversion of its cellular sibling, PrP^{C}, into a likeness of itself? Which other proteins assist this process? Can we inhibit this process? If so, how?
- What is the essence of prion strains, which are operationally defined as variants of the infectious agent capable of retaining stable phenotypic traits upon serial passage in syngeneic hosts? The existence of strains is very well known in virology, but it was not predicted to exist in the case of an agent that propagates epigenetically.
- How do prions reach the brain after having entered the body? Which molecules and which cell types are involved in this process of neuroinvasion? Which inhibitory strategies are likely to succeed?
- The mechanisms of neurodegeneration in spongiform encephalopathies is not understood. Which are the pathogenetic cascades that are activated upon accumulation of disease-associated prion protein and ultimately lead to brain damage?
- What is the physiological function of the highly conserved, normal prion protein, PrP^{C}? The *Prnp* gene encoding PrP^{C} was identified in 1985 (Basler et al., 1986; Oesch et al., 1985), *Prnp* knockout mice were described in 1992 (Büeler et al., 1992), and some PrP^{C}-interacting proteins have been identified (Oesch et al., 1990; Rieger et al., 1997; Yehiely et al., 2002; Zanata et al., 2002). Yet the function of PrP^{C} remains unknown!

The questions described above deserve to be addressed with a vigorous research effort. Their study is likely to yield fundamental insights into the characteristics of these novel and essentially mysterious agents and may yield useful leads for the diagnosis and therapy of prion diseases.

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